IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Oleg Iliich Epshtein
Title of Invention	:	A Medicinal agent and method for curing erectile dysfunction
Date Filed	:	January 22, 2005
Serial No.	:	10/522,650
Examiner	:	Ouspenskii, I.
Art Unit	:	1644
Confirmation No.	:	7546

DECLARATION UNDER 37 CFR 1.132

I, O. I. Epshtein, Dr. Sc, do hereby declare as follows:

1. My name is Dr. Oleg I. Epstein (aka Epshtein). I am a widely recognized scientist in the fields of pharmacology and physiology. I authored over 100 articles in the peer-reviewed journals.

2. The company I lead, Materia Medica Holdings, successfully sells the product covered by the above-identified application 10/522,650. I am the inventor of the '650 application.

3. Attached herewith as Exhibit I is a Report entitled *Sexual Behavior And Erectile Function In Mature Rats With Reduced Erectile Function: The Influence Of 4-week Treatment*, (2007) prepared by Institute of Psychology, University of Tromsoe, an outside vendor retained by Materia Medica to conduct an independent evaluation of the effectiveness of Materia Medica's preparation of homeopathic form of antibodies to NO synthase. The substance of the report is incorporated by reference herein and discussed below in brief.

4. The mice were divided into 5 Groups of 10. Group 1 (control group) was given oral administration of distilled water, administered in one dose: 3 ml/kg daily for 28 days. Mice in Groups 2 and 3 were given oral administration of antibodies to endothelial NO synthase, ultra-low doses (active ingredient of impaza) administered in two doses: 3 ml/kg and 9 ml/kg respectively daily for 28 days. Group 4 was given oral

administration of antibodies to endothelial NO synthase, ultra-low doses (active ingredient of impaza) and sildenafil citrate (Viagra) administered in one dose: 3 ml/kg of impaza and 3 mg/kg sildenafil citrate twice weekly for 28 days. On the days that sildenafil was not given, distilled water was administered. Group 5 was given sildenafil citrate administered in one dose: 3 mg/kg twice weekly. On the days that sildenafil was not given, distilled water was administered. Behavioral testing was performed on days 0 (baseline) and at treatment days 7, 14 and 28.

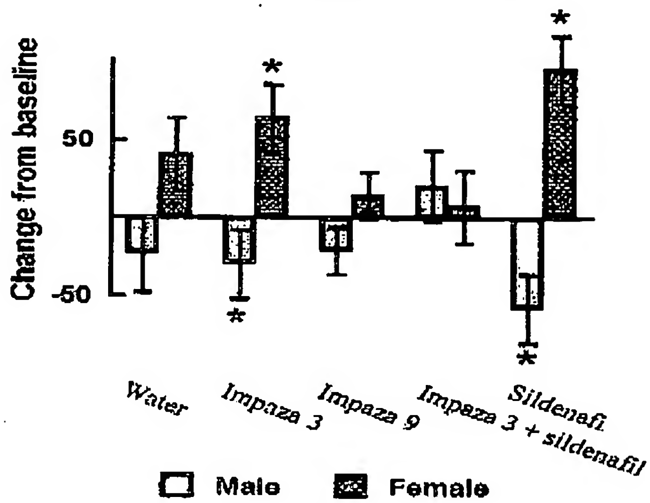
5. The following behavioral parameters were recorded: mount latency; intromission latency; ejaculation latency; post ejaculatory interval; number of mounts and number of intromissions. Sexual motivation was quantified in several ways. Most important for evaluating changes in the sexual incentive value of the receptive female are the preference score (time spent in the female incentive zone/(time spent in the female incentive zone + time spent in the male incentive zone)) and time spent in the female incentive zone. Table 1 below shows that in Fisher 344 rats treatment with sildenafil or Impaza, 9 ml/kg enhanced the intromission ratio at day 28 of treatment Table 2 below suggests that in Wistar rats Impaza 3ml/kg augmented the time present in the female incentive zone between baseline and the test on day 28 of treatment and reduced the time spent in the male incentive zone. Sildenafil had an identical effect. The other treatments were ineffective.

Table 1 - Copulatory behavior in Fisher 344 males at the test performed on day 28 of treatment. Data are mean ± SEM

Behaviour parameter	Treatment				
	Water	Impaza 3	Impaza 9	Impaza 3 + sildenafil	Sildenafil
Mount latency	164 ± 76	180 ± 87	55 ± 11	189 ± 136	258 ± 144
Intromission latency	141 ± 48	223 ± 88	66 ± 16	202 ± 138	246 ± 133
Ejaculation latency	339 ± 52	353 ± 51	416 ± 159	228 ± 95	316 ± 100
Postej. interval	350 ± 48	364 ± 41	315 ± 56	280 ± 14	306 ± 21
N of mounts	15 ± 5	10 ± 4	3 ± 1	9 ± 7	5 ± 3
N of intromissions	5 ± 1	8 ± 2	4 ± 2	2 ± 1	4 ± 1
Intromission ratio	0.25 ± 0.07	0.49 ± 0.06	0.66 ± 0.05*	0.46 ± 0.13	0.61 ± 0.13*

*, different from water, $P < 0.05$, Duncan's multiple range test.

Table 2 - Mean ± SEM change from baseline (in Wistar rats) in time (sec) spent in the male and female incentive zones at day 28 of treatment. * $P < 0.05$(observed value compared to 0 (no change) with a t-test).



6. Attached herewith as Exhibit II, is a Report entitled *Sexual Behavior And Erectile Function In Old Rats: The Influence Of 4-week Treatment*, (2006) prepared by Institute of Psychology, University of Tromsoe, an outside vendor retained by Materia Medica to conduct an independent evaluation of the effectiveness of Materia Medica's preparation of homeopathic form of antibodies to NO synthase. Also attached herewith as Exhibit III, is an article entitled *Sexual Incentive Motivation In Old Male Rats: The Effects Of Sildenafil And A Compound (Impaza) Stimulating Endothelia NO Synthase*, Pharmacology, Biochemistry and Behavior 89 (2008), 209-217. The substance of the report and the article are incorporated by reference herein and discussed below in brief.

7. The mice were divided into 5 Groups of 10. Groups 1 and 2 were given currently used sample (sample 1) containing antibodies to endothelial NO synthase, ultra-low doses (active ingredient of impaza) administered in two doses: 3 ml/kg and 9 ml/kg respectively daily for 28 days. Mice in Group 3 was given experimental sample (sample 2) containing antibodies to endothelial NO synthase, ultra-low doses (active ingredient of impaza) administered in one dose: 3 ml/kg daily for 28 days. Group 4 (control group) was given oral administration of distilled water, administered in one dose: 3 ml/kg daily

for 28 days. Group 5 was given sildenafil citrate administered in one dose: 3 mg/kg twice weekly. On the days that sildenafil was not given, distilled water was administered. Behavioral testing was performed on days 0 (baseline) and at treatment days 7, 14 and 28.

8. Table 3 shows that sample 1 administered in the volume of 3 ml/kg for 4 weeks stimulates sexual motivation in old, sexually inactive male rats. Table 4 shows that that there was a significant difference between the time spent in the receptive female incentive zone than in the male incentive zone only in the group treated with sample 1, 3 ml/kg or with sildenafil. In the other groups, there was no significant difference between the time spent in the vicinity of the male incentive and that spent in the vicinity of the female incentive.

Table 3- Mean ± SEM preference score in 5 groups of male rats at the test of day 28 of treatment. *, difference from no preference, a score of 0.5, P<0.05; +, difference from water, P<0/.05.

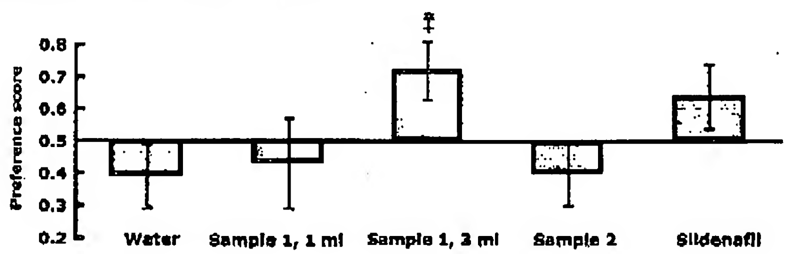
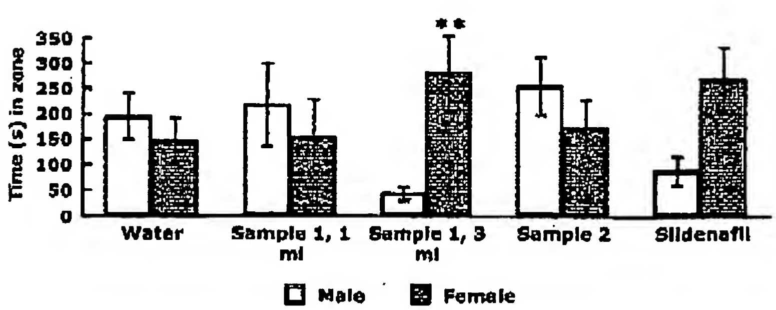


Table 4 – Time spent (sec) in the incentive zones at the test on treatment day 28.



9. The above described data suggest that treatment in Fisher 344 males with Impaza, 9 ml/kg facilitates vaginal penetration through enhanced erection as well as sildenafil; treatment in Wistar rats with 3 ml/kg or sildenafil increase sexual motivation as well as sildenafil; and old rats treated with Impaza 3 ml/kg displayed a preference for the sexually receptive female.

10. In my opinion, the results of the Institute for Psychology study clearly support a conclusion that a preparation based on homeopathic dilution of antibodies NO synthase is statistically far more effective than placebo (water).

11. It is also my opinion that a preparation based on homeopathic dilution of antibodies to NO synthase is at least as effective as or more effective than sildenafil.

12. It is also my opinion that the results of use of Impaza in rat model described in Exhibits I-III would be unexpected by one skilled in the field of erectile dysfunction. In particular, one skilled in the art could not expect, in my opinion, that Impaza will demonstrate results comparable to sildenafil, which is the standard of care for erectile dysfunction.

All statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment; or both, under Section 1001 of Title 18 of the U.S. Code and that such willful false statements may jeopardize the validity of any patent application issuing thereon.

Dated: August 13, 2009

Dr. Oleg I. Epstein

PRECLINICAL STUDY REPORT

Sexual behaviour and erectile function in mature rats with reduced erectile function: The influence of 4-week treatment

Study Director:

Anders Ågmo, Professor

Institute for Psychology, University of Tromsoe, Norway

Study Sponsor:

"Materia Medica Holding" company, Moscow, Russia

First version: 28 August 2007
Final version: 26 November 2007

MAIN OBJECTIVE

Evaluate the efficacy of the tested drug (provided by "Materia Medica Holding" company, Russia) in an animal model of erectile/ sexual dysfunction.

Test substance:
Antibodies to C-terminal fragment of endothelial NO synthase (20 amino acids), ultra-low doses for oral administration (mixture of homeopathic dilutions C12, C30, and C200). The tested substance is an active ingredient of a therapeutic approved in Russia for the treatment of erectile dysfunction (impaza)."

Reference substance:
Sildenafil citrate (selective inhibitor of phosphodiestherase type 5, a standard therapy for erectile dysfunction in humans).

STUDY DIRECTOR'S AUTHENTICATION

I, the undersigned, hereby declare that the work described in this report was performed under my supervision as Study Director and that the final report provides a true and accurate record of the results obtained.

Anders Agmo,
Professor of biological psychology
Study Director

Date: November 26, 2007

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STUDY ORGANISATION

Study Sponsor

The experiments are sponsored by OOO NPF "Materia Medica Holding", a company organised and existing under the laws of Russian Federation.

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127473, Moscow,
Russian Federation
Phone/fax: +7 495 631 24 76 (Research and Development department)
Project manager: Andrey Martyushev-Poklad, M.D., Ph.D.

Test facility

The experiments were carried out in the animal facilities of the Institute of Medical Biology, Faculty of Medicine, University of Tromsoe.

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Personnel

Study Director: Anders Ågmo, Ph.D., Professor.
Work done by: Xi Chu, graduate student.
Animal care and some other assistance: Ragnhild Osnes and Stig Rune Olsen, laboratory technicians.
Date for start of experimental work: 16.04.2007.
Date for completion of experimental work: 03.07.2007.

Archiving

The raw data are kept by Dr. Anders Ågmo at the University of Tromsoe.

Schedule

Numbers refer to weeks of 2007.

Weeks 16 – 17. Acquisition of copulatory experience, familiarization to sexual incentive motivation test environment for the first half of the animals. After 4 tests, the 50 animals with the lowest intromission ratio were selected from those animals that had displayed copulatory behaviour. The other 50 animals were eliminated.

Weeks 18 – 21. Drug treatment started on May 2 (one half of the animals selected from the first group) and on May 3 (the other half of the selected animals from the first group) and ended on May 29 and 30, respectively.
Weeks 20 – 21. Acquisition of copulatory experience, familiarization to sexual incentive motivation test environment for the second half of the animals. After 4 tests, the 50 animals with the lowest intromission ratio were selected from those animals that had displayed copulatory behaviour. The other 50 animals were eliminated.
Weeks 22 – 26. Drug treatment started on June 1 (one half of the selected animals from the second group) and on June 2 (the other half of the selected animals from the second group) and ended on June 28 and 29, respectively.

MATERIALS AND METHODS

Test subjects

1) A total of 100 experimentally and drug naïve, about 4 – 5 months old Wistar rats and 100 experimentally and drug naïve, about 4 – 5 months old Fisher 344 rats, all from B&K, Sollentuna, Sweden were used. The weight of the subjects upon arrival was 347 ± 14 g (mean ± standard error) for the Wistar rats and 320 ± 8 g for the Fisher 344 rats.
2) Eight male rats were used as neutral incentives in the sexual incentive motivation part of the experiment. These males (300 – 400 g) were also of the Wistar strain and bought from B&K, Sollentuna, Sweden.
3) Twenty-four female Wistar rats (300-350 g, B&K, Sollentuna, Sweden) were used as copulation partners. They were ovariectomized under isoflurane anesthesia at least 2 weeks before use and given estradiol benzoate (25 µg, Sigma) 48 hrs before testing and progesterone, 1 mg, about 4 hrs before each session.

The rats were housed in pairs in Macrolon IV cages, in a temperature controlled animal room at +21°C ± 1 °C, at a relative humidity of 55% ± 10% and on a reversed 12 h light/dark cycle (lights on 23:00 - 11:00), with free access to water and food. Standard certified dry pelleted food, rodent low protein, supplied by B&K Universal, Sollentuna, Sweden was used. Tap water was available to the animals ad libitum in Macrolon bottles. The water was checked daily and bottles changed twice a week.

All experimentation was approved by the local laboratory animal care and experimentation committee. The animals were housed according to the rules of European Convention (EC, 1990) and to the rules of National Research Council (NRC, 1996) USA.

Test articles

Hormones used for the induction of sexual receptivity in the females

Crystalline β-estradiol (Sigma, batch 88H3787) and progesterone (Sigma, batch 89H0640) were mixed with peanut oil (Apotekproduksjon, lot 4E090/1) and heated to 60 °C for 24 hrs in order to produce a stock solution. This was diluted in peanut oil to the appropriate concentration (125 μg/ml for estradiol benzoate and 5 mg/ml for progesterone). The steroids were injected s.c. in a volume of 0.2 ml/rat.

Experimental drugs

Test substance:

1) Antibodies to C-terminal fragment of endothelial NO synthase (20 amino acids), ultra-low doses for oral administration (anti-NOS) – active ingredient of Impaza (a therapeutic approved in Russia for the treatment of erectile dysfunction)..

Anti-NOS was provided as a water solution ready for use (no smell, no taste) in 250 ml plastic vials, delivered via DHL and given by gavage once daily (at 9-10 a.m.) for 28 days. Two doses were administered, 3 and 9 ml/kg. Each dose was given to 10 rats of each strain. On the days of tests, anti-NOS was given 1-2 hours before the start of testing.

2) Passive control: Vehicle (distilled water provided by the Physiology Department, University of Tromsø) was given by gavage, 1 ml/rat daily for 28 days (10 rats of each strain). On the days of tests, vehicle was given 1-2 hours before the start of testing.

3) Active control: Sildenafil citrate (Viagra®, Pfizer, batch 5185049NO, obtained from Sykehusapoteket, Tromsoe) was dissolved in distilled water to a concentration of 1 mg/ml immediately before use and given at a dose of 3 mg/kg p.o. twice weekly for 4 weeks (10 rats opf each strain). On the days of tests, sildenafil was given 1-2 hours before testing.

The sildenafil citrate solution was made by thoroughly crushing one tablet of 25 mg in a porcelain mortar and adding 25 ml of distilled water to the resulting powder. The mixture was carefully stirred for about 30 min before gavage was performed.

Methods

As an OECD Test Guideline is not available for the present study, the following protocol has been chosen as the Guideline: Ågmo, A. (1997). Male rat sexual behaviour. Brain Research Protocols, 1(2): 203-209.

The procedures employed here are standard techniques used for analyses of copulatory behaviour and sexual motivation (defined as the urge to seek contact with an individual of the opposite sex). There are many minor variations, such as size and shape of the observation arena, duration of the test, etc.. However, none of these variations have any systematic effect on the behaviour observed. The capacity to achieve vaginal penetration during the test for copulatory

behaviour has been found to be exquisitely dependent on appropriate erection, and constitutes the most sensitive system for evaluating the efficiency of proerectile compounds *in copula*.

Sexual incentive motivation test environment

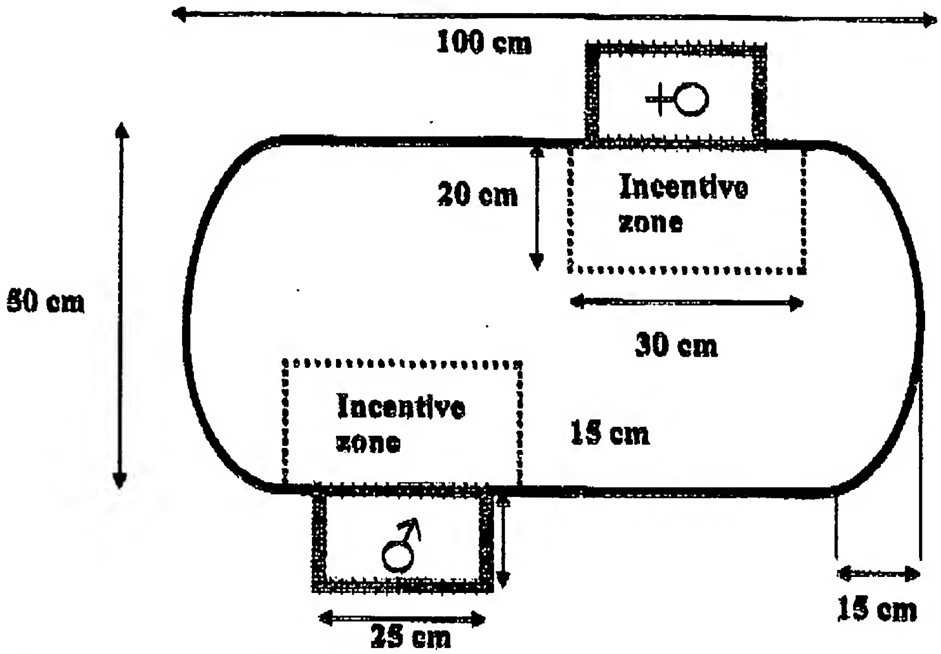

Fig. 1. The apparatus for evaluating sexual incentive motivation. For further details, see text

The test for sexual incentive motivation reveals subtle changes in general arousal, expressed as forward locomotion and speed of movement, in addition to changes in sexual interest. The observation arena is illustrated in Fig. 1. The arena walls and the incentive animals cages were made of sheet steel covered with a black plastic surface. Dark grey polyvinylchloride was used for the floor. The incentive animal cage wall facing the arena was of a 1 x 1 cm stainless steel wire mesh. The apparatus was located in a room adjacent to the animals' room. A video camera was installed above the arena. The camera was connected to a computer. The experimental subject's position was determined online with a videotrack system (Ethovision, Noldus, Wageningen, The Netherlands). An incandescent light bulb provided dim white light (about 5 lux in the arena).

Copulatory behaviour test environment

Black sheet-steel cages (40 x 60 x 40 cm high) with Plexiglas front and glass floor were positioned over a mirror inclined 45 degrees. This allowed for a simultaneous side and ventral view of the copulating male. Tests were recorded on videotape with a 2-camera system connected to a VCR via a multiplexer.

Detailed description of procedures

Habituation of the male rats to sexual incentive motivation tests

The animals were familiarized to the observation arena during 3 sessions of 10 min each. During these sessions, incentive animal cages were empty.

Sexual incentive motivation tests

Before each experimental session the arena and the incentive animal cages were carefully washed with a 0.1 % acetic acid solution. The incentive animals were then placed in their respective cages. About 5 min later the first experimental subject was introduced into the middle of the arena. Immediately thereafter, the experimenter left the room and did not return until just after the end of the 10 min observation period. The subject was then gently removed from the arena, and the following rat was immediately introduced. No cleaning was performed between trials within a session. The position of the incentive animals were semi-randomly changed throughout the experimental session. At the end of every session, half of the animals had had the incentives in one position and the other half in the other. Care was taken to avoid that any single animal had the incentive animals in the same position in more than two consecutive sessions. Spatial location was, therefore, a useless predictor of the state of the incentives. In all experiments, the incentives were a receptive female (Wistar, about 5 months old at the beginning of experiments) and an intact male (Wistar, about 5 months old at the beginning of the experiment). The receptive female had always received the hormone treatment mentioned previously. All incentive animals were sexually inexperienced. For more details of procedure, see Ågmo, 2003, Ågmo et al., 2004.

Tests for copulatory behaviour

Copulatory behaviour was observed in a room separate from the sexual incentive motivation test. To assure that contextual conditioning during copulation could not affect tests for sexual incentive motivation, the copulation test room differed from the incentive motivation test room in several ways. It was brightly lit (about 300 lux in the observation cages), the furniture was different and the general arrangement of the room was also different. For example, the observation cages were located on a table whereas the incentive motivation test arenas were located on the floor.

The male was put into the observation cage about 5 min before a receptive female was introduced. Copulatory behaviour was then observed until the 1st ejaculation. The following behavioural parameters were recorded with in-house software: Mount latency (time from introduction of the female until the first mount with pelvic thrusting), intromission latency (time from introduction of the female until the first mount with vaginal penetration), ejaculation latency (time from the 1st intromission until ejaculation), the postejaculatory interval (time between the

ejaculation and the next intromission), number of mounts, and number of intromissions. The intromission ratio (number of intromissions / (number of mounts + number of intromissions) were also calculated. In case a male displaying intromission did not perform any mount without intromission the intromission ratio is 1 (number of intromissions / (number of intromissions + 0) = 1). This is the most sensitive behavioural measure of erectile functioning. If no mounting occurred, the test was terminated after 15 min. It was also terminated if the ejaculation latency became > 30 min or the postejaculatory interval longer than 15 min. A more extensive description can be found in: Ågmo, 1997.

In addition, the length of the part of the erect penis protruding from the prepuce during mount and/or following withdrawal after intromission or ejaculation was estimated from the video record. From the beginning of a mount with or without intromission/ejaculation until withdrawal the video was advanced frame by frame. The frame where the erection was maximal was always chosen for measurement of the protruding penis. Measurement was not possible at every mount or intromission because of an unsatisfactory view. Nevertheless, in most sexually active animals at least 5 erections during mount and another 5 after intromission were measurable. In case that the subject displayed more than 5 mounts and intromissions, only the first 5 were measured. The erection observed after ejaculatory withdrawal was measured whenever possible. The mean penis length for mount and intromission was then calculated for each animal at each test. This mean was used for statistical analysis. An arbitrary measurement unit was employed (mm on the projection screen), but all values can be transformed to actual penis length.

Design

The following five groups of 10 rats from each strain were employed:

Group 1. Distilled water. Daily oral administration (gavage). Volume was 3 ml/kg.
Groups 2 and 3. Impaza 3 and 9 ml/kg, respectively.
Group 4. Impaza 3 ml/kg daily + sildenafil 3 mg/kg twice weekly. On days when sildenafil were not given, distilled water was administered.
Group 5 Sildenafil, 3 mg/kg p.o. Twice weekly. On days when sildenafil were not given, distilled water was administered. The sildenafil dose of 3 mg/kg p.o. was intermediate between doses that earlier had been found effective on male rat sexual behaviour (Ferrari et al., 2002; Giuliani et al., 2002; Ottani et al., 2002). It was far above the dose needed to potentiate the effects of apomorphine on intracavernous pressure (0.1 mg/kg; Andersson et al., 1999). However, that study had employed intravenous administration and was, therefore, not directly comparable.

After preliminary testing of the 100 Fisher 344 and the 100 Wistar rats, all subjects that did not display copulatory behaviour were eliminated. Of the remaining animals, the 50 in each strain showing the lowest intromission ratio were selected for the experiment. For the selection procedure, males showing no sexual behaviour at all were assigned an intromission ratio of 1 and immediately eliminated. Among the remaining animals, those having displayed copulatory behaviour at only one test were excluded. Then, the 50 animals of each strain having the lowest

intromission ratio were selected. The animals selected for the drug treatments had an intromission ratio of 0.16 ± 0.01 (mean ± SEM) while the discarded animals had a ratio of 0.24 ± 0.04. Experimental tests of the selected animals were performed on days 0 (baseline test) and on treatment days 7, 14 and 28. On test days, the compounds were administered 1 – 2 h before observation. In group 4, sildenafil was administered about 5 min before impaza.

After the last behavioural test, the animals were euthanized and penile tissue immediately removed, frozen in liquid nitrogen and stored at –70 °C for future analyses.

Records

The following primary records (raw data) were made in the course of the study:

1) Experimental register (journal/ log) describing all procedures and manipulations performed with animals in the course of the study (day by day).
2) Videotapes for tests for copulatory behaviour – for all animals were made and provided to the Sponsor as raw data; the coordinates of the experimental rats' position in the incentive motivation test environment, recorded with a frequency of 5 Hz, are stored on the lab computer's hard disk, and can be made available at any moment.
3) Transcripts for all videotapes with all parameters mentioned above for each rat (*electronic format*)
4) Lists of all parameters derived from p.3 in the form of electronic tables designed for data processing and statistics (*electronic format*).

The following raw data are stored by the University: The electronic files generated by the video track system; the electronic files generated by the copulatory behaviour observation program.

Two originals of Study Reports are sent to the Sponsor, one original of Study report is stored by the University.

Data processing and statistics

General

Comparisons between Wistar and Fisher 344 rats were based on the data obtained at the baseline test, before drug treatments had been initiated. The Wistar and Fisher 344 control groups were also compared both at the baseline test and at the test performed at day 28 of treatment. The analyses of treatment effects were made separately for Wistar and Fisher 344 rats. Otherwise, the analyses would have become exceedingly complex and difficult to interpret.

Sexual motivation was quantified in several ways. Most important for evaluating changes in the sexual incentive value of the receptive female are the *preference score* (time spent in the female incentive zone/(time spent in the female incentive zone + time spent in the male incentive zone)) and *time spent in the female incentive zone*. There need to be a statistically significant change on both parameters if an effect on sexual motivation is to be considered. A double criterion is needed in order to avoid false positive effects. An increased preference score may be a result of either increased time in the female zone or reduced time in the male zone or a combination of both. However, reduced time in the male zone without a concomitant increase in

time in the female zone does not necessarily indicate enhanced sexual incentive motivation. At the same time, an increase in the time spent in the female zone could be a consequence of increased attractivity of any incentive animal and is therefore not a sufficient indicator of increased sexual incentive motivation. Similar arguments could be made for reduced sexual incentive motivation. The use of both criteria (change in preference score *and* a corresponding change in time spent in the female zone) avoids the pitfalls of them when used singly.

Comparisons between Wistar and Fisher 344 rats

All parameters of sexual motivation and penis length as well as most of sexual behaviours were compared with the *t*-test for independent groups. The proportion of subjects displaying mount, intromission or ejaculation was evaluated with the Fisher exact probability test.

Comparisons of treatments

The preference score was analysed with two-factor ANOVA with repeated measures on one factor, the between-groups factor being treatment and the within-groups factor being test. The time spent in the incentive zones was evaluated by three-factor ANOVA with repeated measures on two factors, the within group factors being incentive (male, female) and test and the between group factor being treatment. Indices of ambulatory activity at all tests were analysed as the preference score, while the number of visits to the incentives were analysed like the time spent in the incentive zones.

Data from the copulatory behaviour tests were analyzed in several ways. The proportion of subjects displaying mount, intromission or ejaculation at each treatment was evaluated with the chi-square test. The number of mounts and intromissions as well as the latencies, intromission ratio and penis length were compared with ANOVA.

In addition to the actual data, the change from baseline was evaluated in each parameter. The value obtained at baseline was simply subtracted from the value obtained at later tests. This procedure allows for a sensitive analysis of changes, corrected for any group differences at baseline. It is commonly used in pharmacological and behavioural studies. Data from these analyses are only reported when they offer information different from that obtained through analyses of the uncorrected data.

In cases where the parametric analyses yielded unclear results (because of borderline significance) or when data might be suspected to be unsuitable for such analyses because of substantial deviations from normality or nonhomogeneous error variances, pairwise nonparametric tests were performed in addition to the parametric tests. The Wilcoxon test was used for intratreatment comparisons.

DEVIATIONS FROM THE STUDY PROTOCOL

None

RESULTS

1. COMPARISONS BETWEEN FISHER 344 AND WISTAR MALES

1.1 Sexual incentive motivation

The Fisher 344 strain had a higher preference score ($t(98) = 2.89$, $P < 0.01$) and spent more time in the vicinity of the sexually receptive female ($t(98) = 2.92$, $P < 0.001$) than the Wistar strain did at the baseline test. This evidently suggests a more intense sexual incentive motivation. To the contrary, the Wistar strain made a larger number of visits to both the male ($t(98) = 4.93$, $P < 0.001$) and female ($t(98) = 2.97$, $P < 0.01$) incentives, moved a larger distance ($t(98) = 5.81$, $P < 0.001$), moved faster while moving ($t(98) = 3.55$, $P < 0.001$) and spent more time moving ($t(98) = 2.11$, $P < 0.05$) than the Fisher 344 rats. Thus, all indices of ambulatory activity suggest that the Wistar rats are more active than the Fisher 344.

When the 10 animals in each control group were compared at the baseline test, a similar pattern of results became evident. However, the difference in preference score between Fisher 344 and Wistar rats failed to reach significance ($t(18) = 2.08$, $P = 0.052$). When the data from the test performed at day 28 of treatment were used, results were almost identical to those obtained at the baseline test. It seems, then, that the differences between strains are stable over time and tests.

Data from the baseline test are shown in Fig. 2 and the control group data are displayed in Figs 3 and 4.

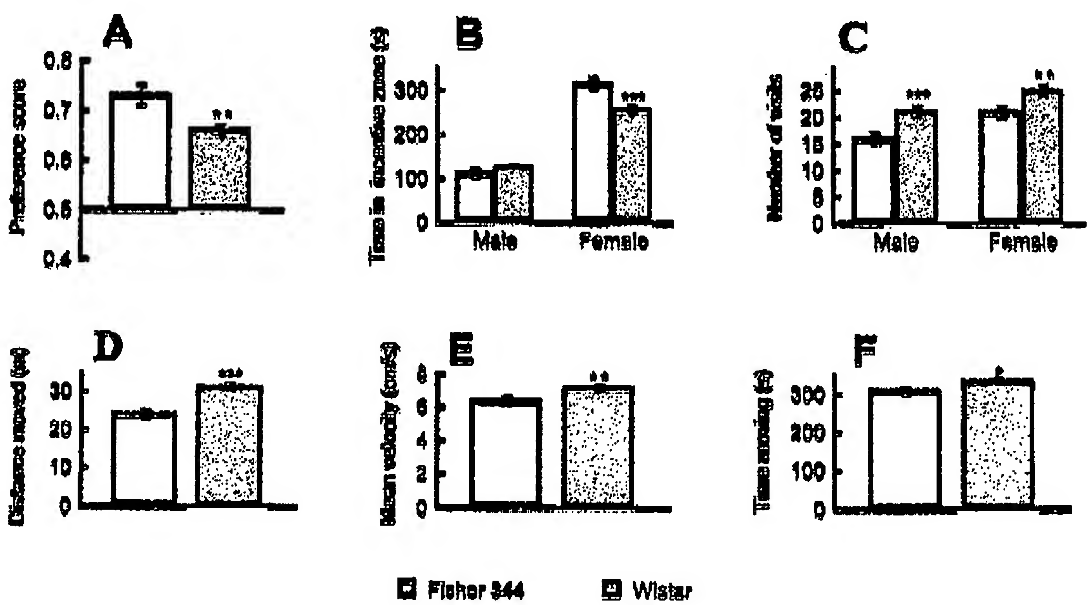

Figure 2. Sexual incentive motivation and ambulatory activity in male Fisher 344 and Wistar rats at the baseline test. **, different from Fisher 344, $P < 0.01$; ***, $P < 0.001$. N = 50 per strain.

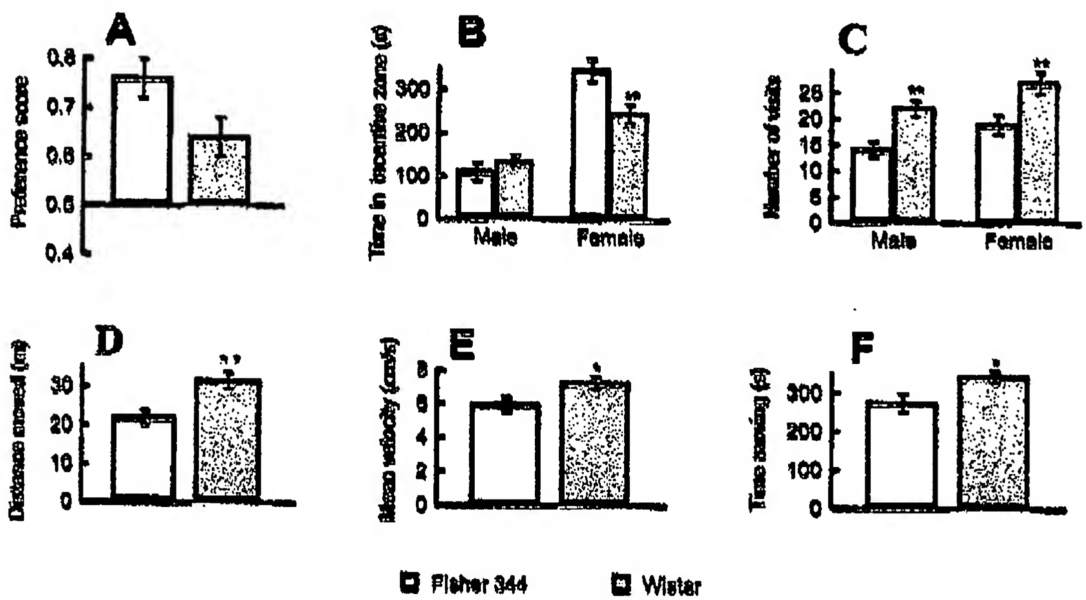

Figure 3. Sexual incentive motivation and ambulatory activity in male Fisher 344 and Wistar control rats at the baseline test. *, different from Fisher 344, $P < 0.05$; **, $P < 0.01$. N = 10 per strain.

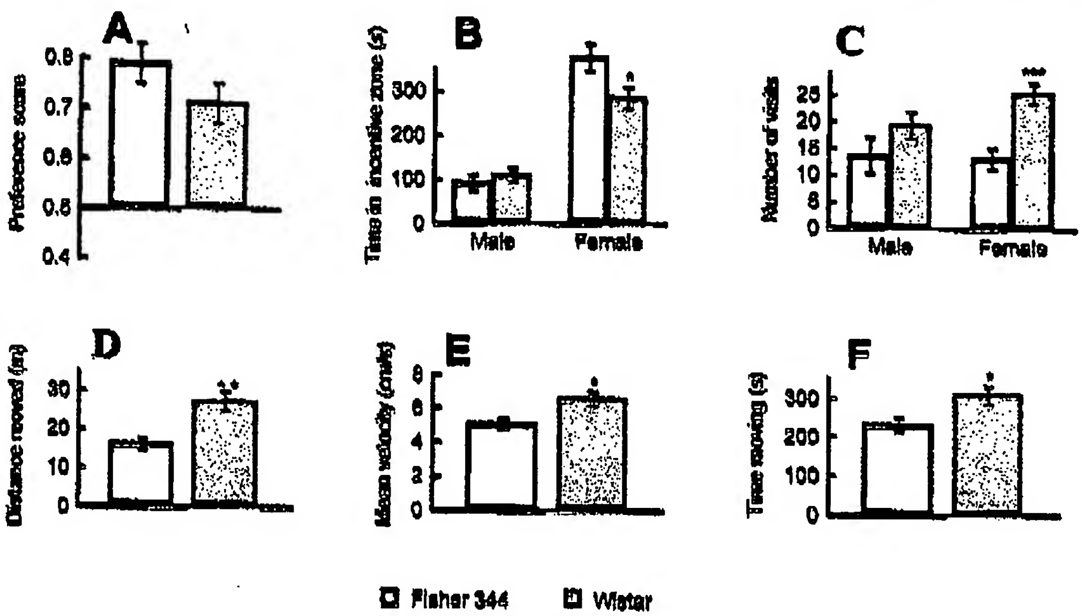

Figure 4. Sexual incentive motivation and ambulatory activity in male Fisher 344 and Wistar control rats at the test performed on day 28 of treatment. *, different from Fisher 344, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. N = 10 per strain.

1.2 Copulatory behaviour

As can be seen in Table 1, there were only minor differences in copulatory behaviour between Fisher 344 and Wistar males. The proportion of animals displaying mount, intromission and ejaculation did not differ. In fact, the only significant difference between strains is found in the number of mounts, where the Wistar rats made far more than the Fisher 344 ($t(98) = 3.41$, $P < 0.01$). The larger number of mounts displayed by the Wistar rats, without any accompanying difference in the number of intromissions, means that the intromission ratio should be lower in the Wistar rats. However, the difference was only of borderline significance.

When the control groups are compared at the baseline test, no significant difference is obtained between the strains. The means shown in Table 2 suggests that the Wistar rats still make more mounts and have a lower intromission ratio, but the absence of significance would suggest that those differences are not particularly reliable. This is confirmed by the data from the test performed at day 28 of treatment (Table 3). There, the Wistar rats make a larger number of intromissions ($t(18) = 2.28$, $P < 0.05$) and have a longer ejaculation latency ($t(18) = 2.44$, $P < 0.05$) than the Fisher 344 rats while the differences seen at the baseline test are absent.

Table 1. Comparison between Fisher 344 and Wistar males at the pretest. All 50 animals from each strain are included. Data are mean ± SEM.

Behaviour parameter	Strain	
	Fisher 344	Wistar
Mount latency	152 ± 28	121 ± 26
Intromission latency	133 ± 29	131 ± 25
Ejaculation latency	448 ± 35	500 ± 52
Postejaculatory interval	351 ± 17	318 ± 21
Number of mounts	13 ± 2	32 ± 5**
Number of intromissions	7 ± 1	8 ± 1
Intromission ratio	0.37 ± 0.04	0.26 ± 0.03[a]

**, different from Fisher 344, $P < 0.01$, t-test. [a], borderline significance, $P = 0.051$ ($t_{(87)} = 1.979$.

Table 2. Comparison between Fisher 344 and Wistar males at the baseline test. Control animals only. Data are mean ± SEM.

Behaviour parameter	Strain	
	Fisher 344	Wistar
Mount latency	140 ± 81	60 ± 15
Intromission latency	45 ± 13	62 ± 16
Ejaculation latency	547 ± 94	589 ± 198
Postejaculatory interval	371 ± 48	351 ± 59
Number of mounts	15 ± 2	25 ± 8
Number of intromissions	8 ± 2	7 ± 2
Intromission ratio	0.30 ± 0.08	0.22 ± 0.09

Table 3. Comparison between Fisher 344 and Wistar males at day 28 of treatment. Control animals only.

Behaviour parameter	Strain	
	Fisher 344	Wistar
Mount latency	163 ± 76	67 ± 32
Intromission latency	141 ± 48	92 ± 32
Ejaculation latency	339 ± 52	641 ± 96*
Postejaculatory interval	349 ± 48	304 ± 19
Number of mounts	15 ± 5	32 ± 10
Number of intromissions	5 ± 1	10 ± 2*
Intromission ratio	0.25 ± 0.07	0.35 ± 0.10

*, different from Fisher 344, $P < 0.05$, t-test.

1.3 Penis length

As can be observed in Table 4, there was a minor difference between Fisher 344 and Wistar rats with regard to penis length after intromission at the baseline test. When the penis length observed while mounting was compared to that observed after intromission or ejaculation, it was found to be shorter, both in Fisher 344 and Wistar rats. In the Fisher rats, the penis length recorded after ejaculation was superior to that recorded after intromission.

Table 4. Comparison of penis length at mount, intromission and ejaculation in Fisher 344 and Wistar males at the baseline test.

Behaviour parameter	Strain	
	Fisher 344	Wistar
Penis length at mount	3.19 ± 0.10	3.04 ± 0.11
Penis length at intromission	4.03 ± 0.11¶	3.68 ± 0.13*¶
Penis length at ejaculation	4.97 ± 0.16$^{¶\#}$	4.54 ± 0.24¶

*, different from Fisher 344, $P < 0.05$, t-test. ¶, different from mount, $P < 0.05$; #, different from intromission, $P < 0.05$. Within strains comparisons were made with repeated measures one-factor ANOVA. N = 25 for the Fisher 344 strain and 13 for the Wistar strain for this analysis.

The penis length in the control group was then analyzed at baseline and at the test performed on day 28 of treatment. Again, the Wistar rats had a shorter penis after intromission than the Fisher 344. As was the case when all animals were included in the analysis, penis length after ejaculation was superior to that after intromission which in turn was superior to that after mount. In the Wistar strain no such comparison was possible since penis length after ejaculation could be determined for only 1 animal. At day 28 of treatment, there was no difference between Fisher 344 and Wistar control animals. Furthermore, the differences between mount, intromission and ejaculation that were observed at baseline had now disappeared in the Fisher 344 rats. To the contrary, the Wistar rats had a longer penis after ejaculation and intromission than after mount. Data are illustrated in Tables 5 and 6.

Table 5. Comparison of penis length at mount, intromission and ejaculation in Fisher 344 and Wistar males at the baseline test. Control animals only.

Behaviour parameter	Strain	
	Fisher 344	Wistar
Penis length at mount	3.22 ± 0.19	3.20 ± 0.28
Penis length at intromission	4.17 ± 0.21[§]	3.35 ± 0.30*
Penis length at ejaculation	5.17 ± 0.31[§#]	6.00[a]

*, different from Fisher 344, $P < 0.05$, *t*-test. [a], only one animal displayed ejaculation in this group. [§], different from mount, $P < 0.05$; [#], different from intromission, $P < 0.05$. Within strains comparisons were made with repeated measures one-factor ANOVA. N = 6 for the Fisher 344 strain. No analysis could be performed on the Wistar strain.

Table 6. Comparison of penis length at mount, intromission and ejaculation in Fisher 344 and Wistar males at the test performed on day 28 of treatment. Data are mean ± SEM. Control animals only.

Behaviour parameter	Strain	
	Fisher 344	Wistar
Penis length at mount	3.43 ± 0.18	3.47 ± 0.25
Penis length at intromission	4.12 ± 0.34	4.17 ± 0.27[§]
Penis length at ejaculation	4.60 ± 0.68	4.83 ± 0.40[§]

[§], different from mount, $P < 0.05$. Within strains comparisons were made with repeated measures one-factor ANOVA. N = 6 for the Fisher 344 strain as well as for the Wistar strain.

2. COMPARISONS BETWEEN TREATMENTS

2.1 Fisher 344

2.1.1 Sexual incentive motivation

2.1.1.1 Preference score

The preference score obtained at the 4 tests (pretest and 3 tests during treatment) in the 5 groups is illustrated in Fig. 5. Data were evaluated with a two-factor mixed ANOVA with treatment as the between groups factor and test as within groups factor. There was no significant main effect of treatment ($F(4,45) = 1.07$, NS) or of test ($F(3,135) = 0.72$, NS) and there was no interaction treatment x test ($F(12,135) = 0.57$, NS). Data are shown in Figure 5. Thus, the treatments failed to affect approach to a sexually receptive female. Likewise, the repeated testing did not modify the intensity of approach.

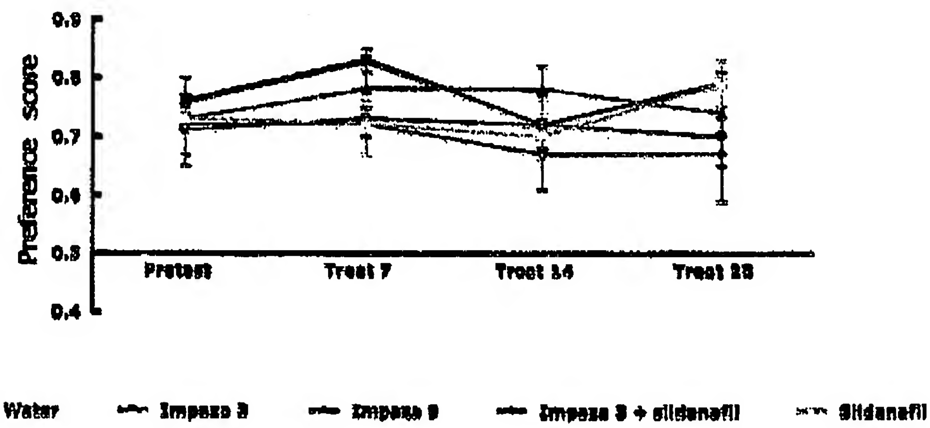

Figure 5. Mean ± S.E.M. preference score in the different treatments of male Fisher 344 rats at the baseline and after 7, 14 and 28 days of treatment.

All groups had a preference score significantly above 0.5, meaning that they always spent more time in the vicinity of the sexually receptive female than in the vicinity of the male incentive (all Ps < 0.01).

When the difference in preference score between baseline and the tests performed at days 7, 14 and 28 of treatment was analyzed, it turned out that there was no effect.

2.1.1.2 Time spent with the receptive female vs. the intact male

When the time spent in the incentive zones (intact male and sexually receptive female) at the 4 tests (pretest and 3 tests during treatment) in the 5 groups of Fisher 344 rats was evaluated with a three-factor mixed ANOVA there was no significant main effect of treatment ($F(4,45) =$

1.33, NS) or of test ($F(3,135) = 0.63$, NS). The incentives (male vs. receptive female) differed ($F(1,45) = 236.95$, $P < 0.001$). There was no incentive x treatment ($F(4,45) = 1.06$, NS) or test x incentive ($F(3,135) = 0.56$, NS) interaction. To the contrary, the interaction test x treatment was significant ($F(12,135) = 1.86$, $P < 0.05$). The three-way interaction test x incentive x treatment was not significant ($F(12,135) = 0.67$, NS). For readability, the illustration of the data is made in two figures, one for the time spent in the male incentive zone (Fig. 6) and another for the time spent in the receptive female incentive zone (Fig. 7).

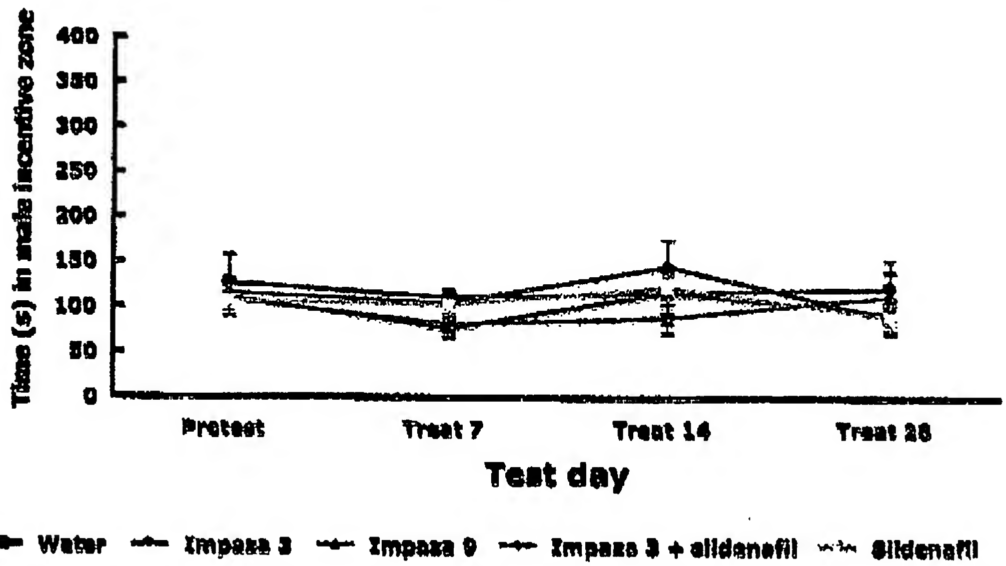

Figure 6. Mean ± S.E.M. time (sec) spent in the male incentive zone in the different treatments of male Fisher 344 rats at the baseline and after 7, 14 and 28 days of treatment.

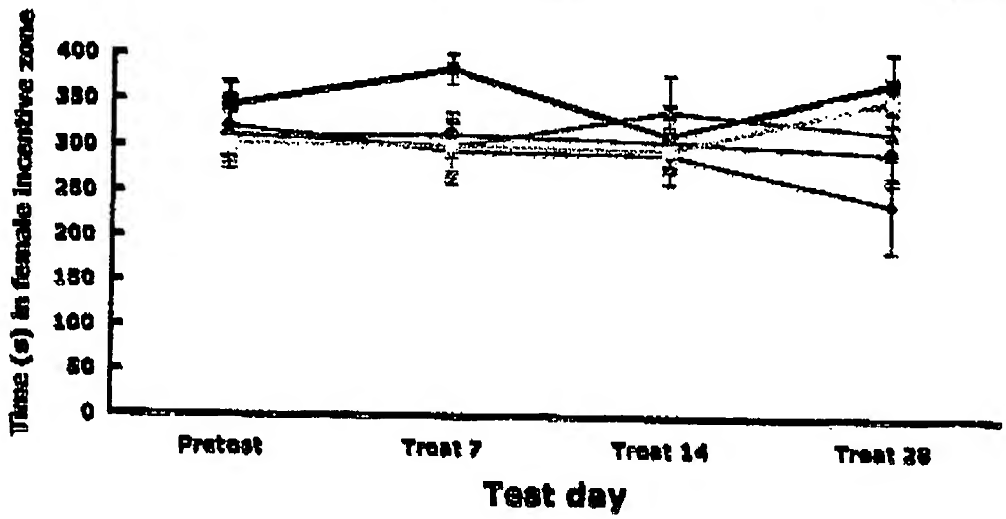

Figure 7. Mean ± S.E.M. time (sec) spent in the female incentive zone in the different treatments of male Fisher 344 rats at the baseline and after 7, 14 and 28 days of treatment.

By examining the Figs. 6 and 7 it can be concluded that the interaction between incentive and treatment is due to a slight decrease in the time spent in the vicinity of the male and a corresponding increase in the time spent in the vicinity of the female in some groups, viz. the control and sildenafil groups. It is difficult to imagine that the interaction represents anything more than a spurious effect.

3.1.1.3 Number of visits to the incentive animals

Three-factor mixed ANOVA of the number of visits to the incentive animals at the 5 test occasions showed a main effect of test ($F(3,135) = 3.57$; $P < 0.05$). There was also an effect of incentive ($F(1,45) = 51.41$; $P < 0.001$) but the treatment effect failed to reach significance ($F(4,45) = 2.18$, NS). The interactions test x treatment ($F(12,135) = 1.09$, NS), and incentive x treatment ($F(4,45) = 2.45$, NS) were nonsignificant. This was also the case for the interactions test x incentive and test x incentive x treatment ($F(3,135) = 0.10$, NS and $F(12,135) = 1.64$, NS, respectively). These results show that none of the treatments affected the number of visits to the incentives. In fact, the only effect obtained was that the number of visits decreased somewhat irregularly with repeated testing and that the subjects made more visits to the receptive female than to the male after all treatments at all tests. For readability, data are illustrated in two figures, one for the number of visits to the male incentive (Fig. 8) and one for visits to the female incentive (Fig. 9).

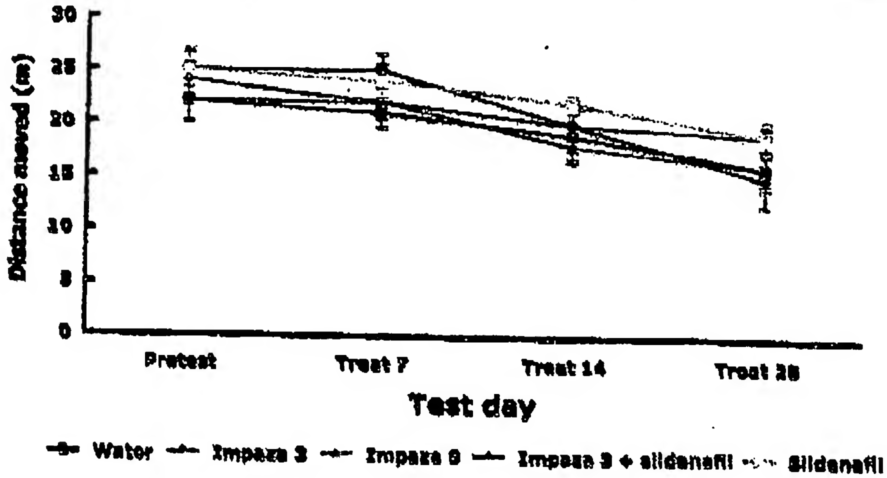

Figure 10. Ambulatory activity expressed as distance moved (in meters) during the sexual incentive motivation test in 5 groups of male Fisher 344 rats at 4 tests.

Similar results were obtained when another indicator of motor function, the mean velocity of movement while moving, was analyzed. There was no effect of treatment, ($F(4,45) = 2.02$, NS) but there was an effect of test ($F(3,135) = 22.76$, $P < 0.001$). The interaction treatment x test turned out to be nonsignificant ($F(12,135) = 0.41$, NS). It is again concluded that there was no treatment effect on velocity of movement but there was a progressive reduction with repeated testing. Data are illustrated in Fig. 11.

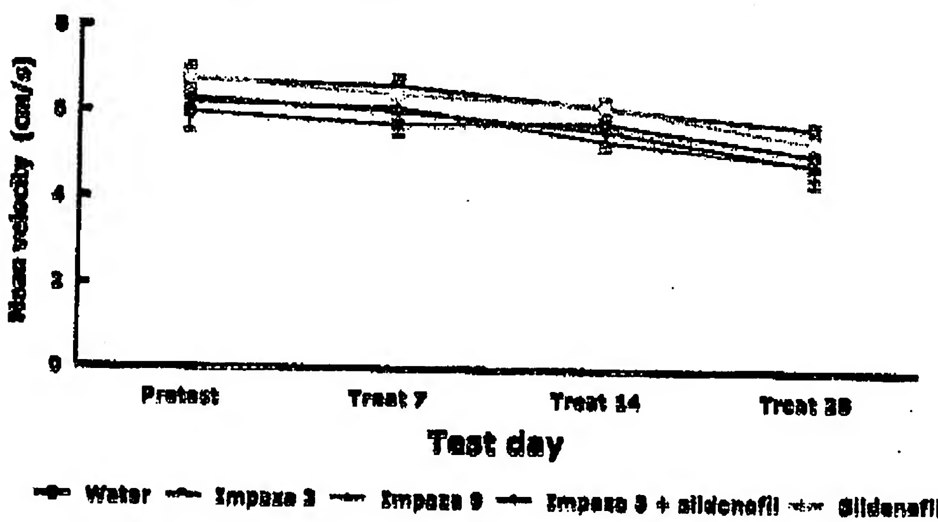

Figure 11. Ambulatory activity expressed as mean velocity of movement while moving (in cm/s) during the sexual incentive motivation test in 5 groups of male Fisher 344 rats at 4 tests.

Finally, the time spent moving) was evaluated. Here there was a difference between treatments ($F(4,45) = 3.07$, $P < 0.05$). *Post hoc* comparisons revealed that the group treated with Impaza, 3 ml/kg, spent more time moving than the other groups. An examination of the data (see Fig. 12) suggests that this difference is not due to drug treatment. In fact, already at the baseline test the Impaza 3 ml/kg group spent much time moving. Furthermore, the interaction treatment x test was not significant ($F(12,135) = 0.91$, NS), reinforcing the notion that treatment was not the cause of the fact that the Impaza 3 ml/kg group spent more time moving. The tests differed ($F(3,135) = 16.12$, $P < 0.001$). Again, there was a progressive decline in the time spent moving.

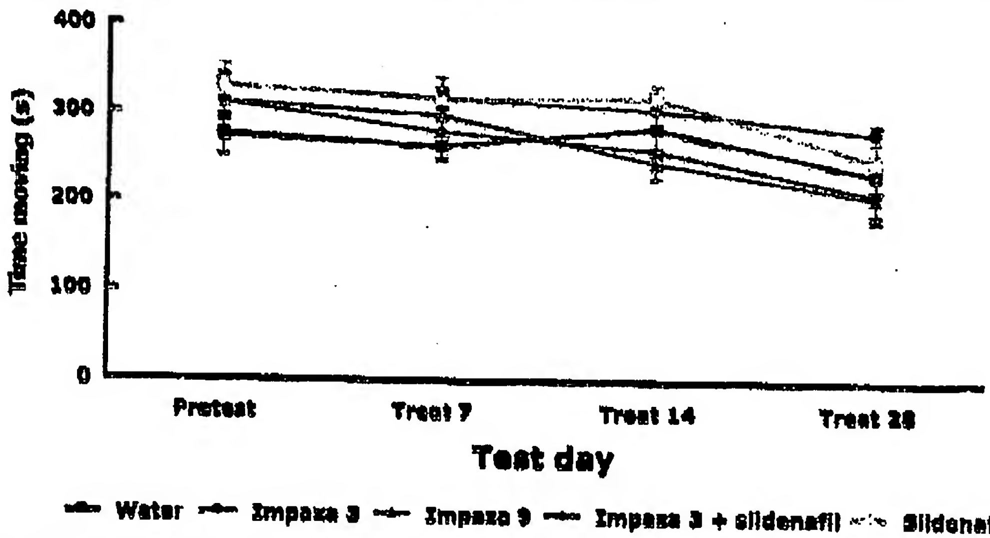

Figure 12. Ambulatory activity expressed as time not moving during the sexual incentive motivation test in 5 groups of male Fisher 344 rats at 4 tests.

2.1.2 Copulatory behaviour

Chi-square tests revealed that the treatment groups did not differ with regard to the proportion of animals displaying at least one mount, intromission, or ejaculation at any test. There is no sign of any drug effect. Data are illustrated in Figures 13 – 15.

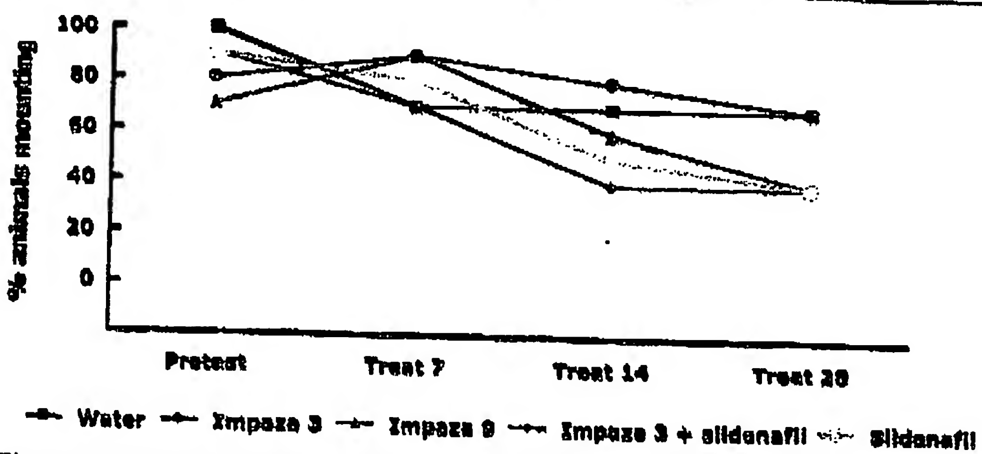

Figure 13. Proportion (expressed as percent) of animals displaying at least one mount at the 4 tests.

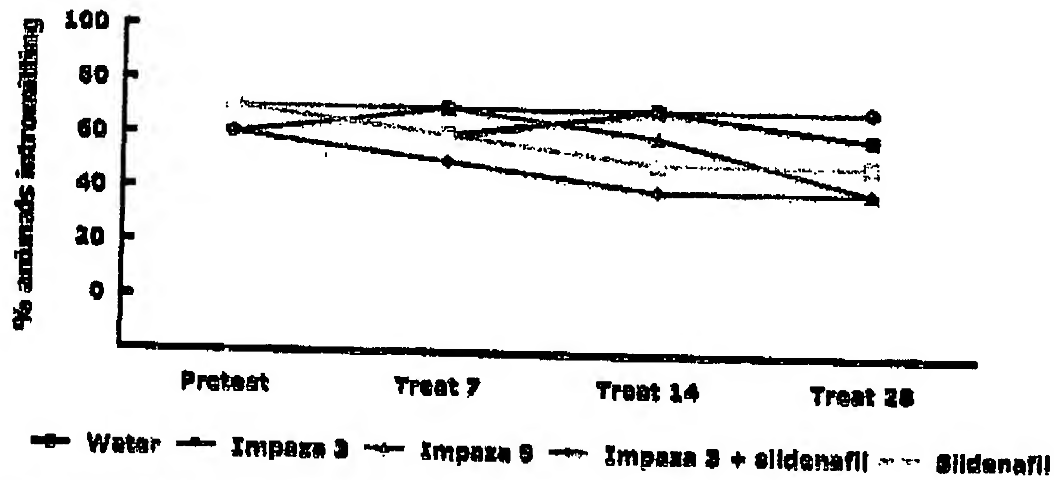

Figure 14. Proportion (expressed as percent) of Fisher 344 rats displaying at least one intromission at the 4 tests.

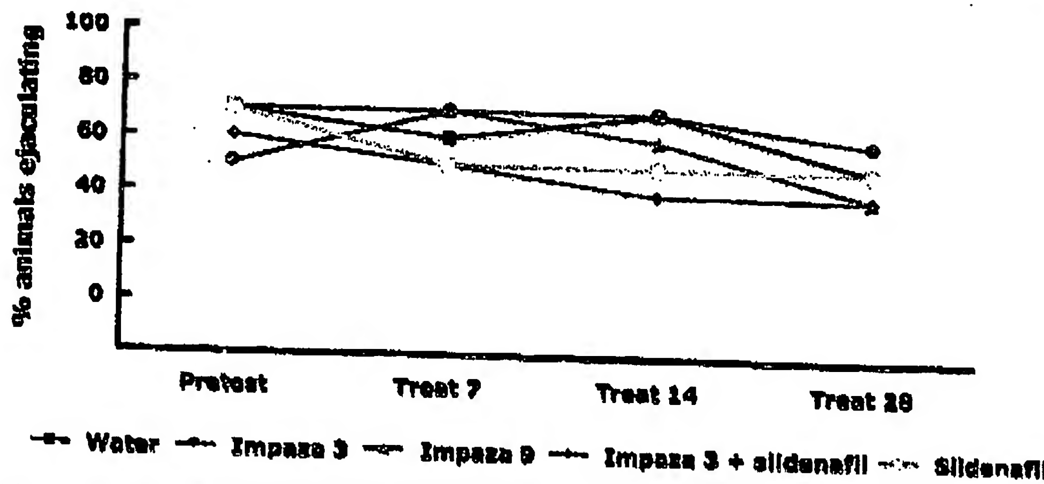

Figure 15. Proportion (expressed as percent) of Fisher 344 displaying ejaculation at the 4 tests.

A two-factor mixed ANOVA of the number of mounts displayed in each treatment group at each test revealed a significant difference between treatments ($F(4,45) = 2.77$, $P < 0.05$). There was no effect of test ($F(3,135) = 2.33$, NS) and no interaction treatment x test ($F(12,1359 = 0.28$, NS). Post hoc comparisons of the data revealed that the control group made more mounts than the group given Impaza, 9 ml/kg, and the one given sildenafil, 3 mg/kg. Inspection of the data reveals that the animals in those latter groups made few mounts, particularly at the tests performed at days 14 and 28 of treatment. Data are illustrated in Fig. 16.

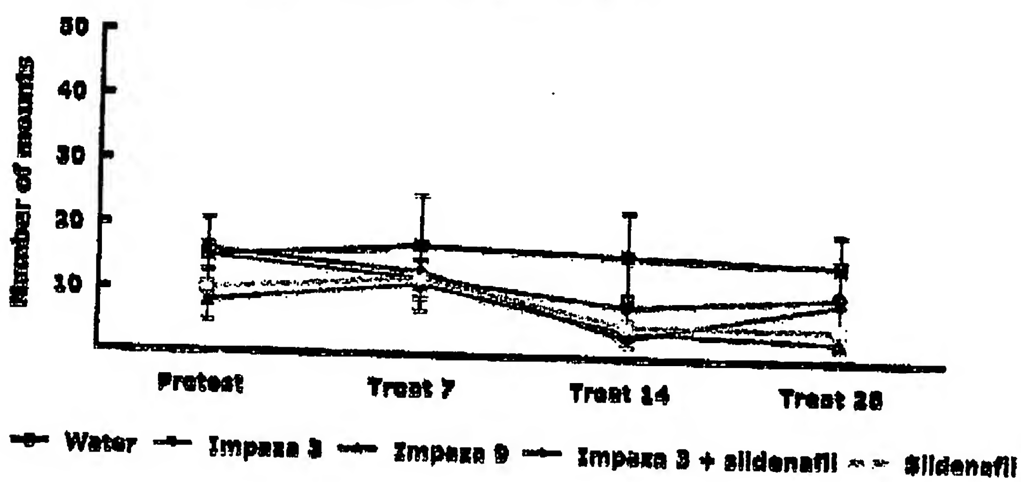

Figure 16. Number of mounts (mean ± S.E.M.) displayed by Fisher 344 rats at baseline and after 7, 14 and 28 days of treatment.

When a similar analysis was performed for the number of intromissions, it turned out that there was no treatment effect ($F(4,45) = 0.55$, NS). To the contrary, there was a difference between tests ($F(3,135) = 4.16$, $P < 0.01$) but no interaction treatment x test ($F(4,135) = 0.70$, NS).

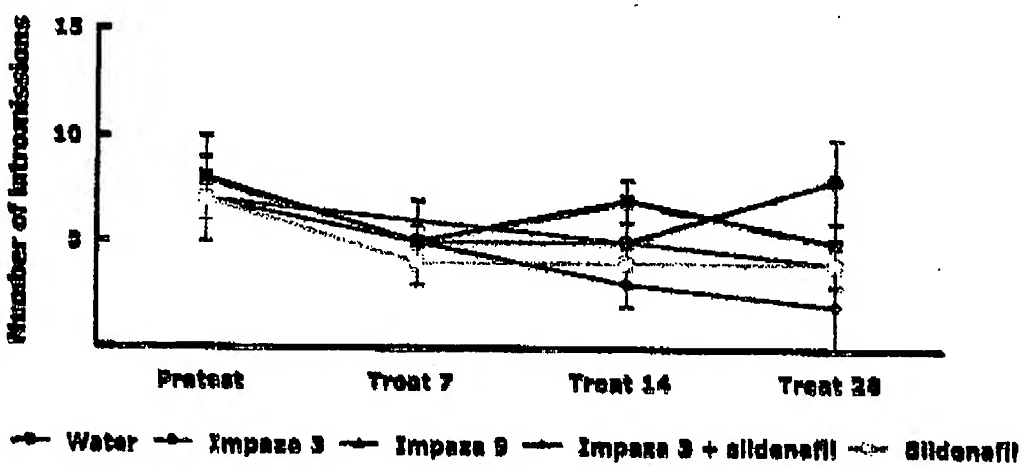

Figure 17. Number of intromissions (mean ± S.E.M.) displayed by Fisher 344 rats at baseline and after 7, 14 and 28 days of treatment.

As can be seen in Fig. 17, the number of intromissions appeared to get reduced with repeated testing, at least in the groups treated with Impaza, 9 ml/kg, Impaza 3 ml/kg + sildenafil and sildenafil. However, since the interaction test treatment was not significant, these comments should be interpreted with caution.

The other measures of sexual behaviour could not be obtained from every animal at every test. Obviously, the latencies cannot be recorded if the behaviour does not occur, and the intromission ratio cannot be calculated in animals displaying neither mount nor intromission. Repeated measures analyses of these data are, therefore, useless. Therefore, a separate analysis was performed for each test day. The treatments were compared with one-factor ANOVA. At baseline, there was no group difference at all (all $Ps > 0.36$). This was also the case for the tests performed at days 7 and 14 of treatment ($Ps > 0.16$ at day 7, and > 0.10 at day 14). On the test performed at day 28 of treatment, however, there was a difference between treatments with regard to intromission ratio ($F(4,22) = 3.46$, $P < 0.05$). *Post hoc* test revealed that the groups treated with Impaza, 9 ml/kg, and sildenafil, 3 mg/kg, had a larger intromission ratio than the control group. Data from this test are illustrated in Table 7.

Table 8. Penis length in Fisher 344 males at the test performed on day 28 of treatment. Data are mean ± SEM.

Behaviour	Treatment				
	Water	Impaza 3	Impaza 9	Impaza 3 + sildenafil	Sildenafil
Mount	0.34 ± 0.02	0.32 ± 0.01	0.37 ± 0.03	0.36 ± 0.03	0.37 ± 0.03
Intromission	0.41 ± 0.03	0.38 ± 0.02	0.42 ± 0.03	0.43 ± 0.02	0.45 ± 0.02
Ejaculation	0.46 ± 0.07	0.50 ± 0.02	0.50 ± 0.08	0.52 ± 0.05	0.52 ± 0.05

Notes: Length is expressed in arbitrary units (mm on the projection screen).

2.2 Wistar

2.2.1 Sexual incentive motivation

2.2.1.1 Preference score

The preference score obtained at the 4 tests (pretest and 3 tests during treatment) in the 5 groups is illustrated in Fig. 18. Data were evaluated with a two-factor mixed ANOVA with treatment as the between groups factor and test as within groups factor. There was no significant main effect of treatment ($F(4,45) = 0.58$, NS) while the effect of test was highly significant ($F(3,135) = 10.81$, $P < 0.001$). This was also the case with the interaction treatment x test ($F(12,135) = 2.61$, $P < 0.01$). As can be seen in Fig. 18, the preference score increased in all groups with repeated testing. This is manifested in the significant difference between tests. Furthermore, the group given sildenafil appears to show a more marked increase than the control group. This may be the cause of the significant interaction. However, tests for simple main effect of treatment at each test revealed that the treatment groups differed only at the test performed on day 14 of treatment ($F(4,45) = 2.65$, $P < 0.05$). *Post hoc* tests showed that none of the treatments differed from control, unfortunately. At day 28, there was no significant group difference ($F(4,45) = 1.49$, NS).

The effect of test was then evaluated within each treatment. Interestingly, the preference score increased in the groups given Impaza 3 and 9 ml/kg as well as in the group treated with sildenafil ($F(3,135) = 3.53$, $P < 0.05$; $F(3,135) = 5.28$, $P < 0.01$; $F(3,135) = 8.63$, $P < 0.001$, respectively). There was no increase with repeated testing in the control group ($F(3,135) = 1.26$, NS) or in the group treated with Impaza 3 ml/kg + sildenafil ($F(3,135) = 2.52$, NS).

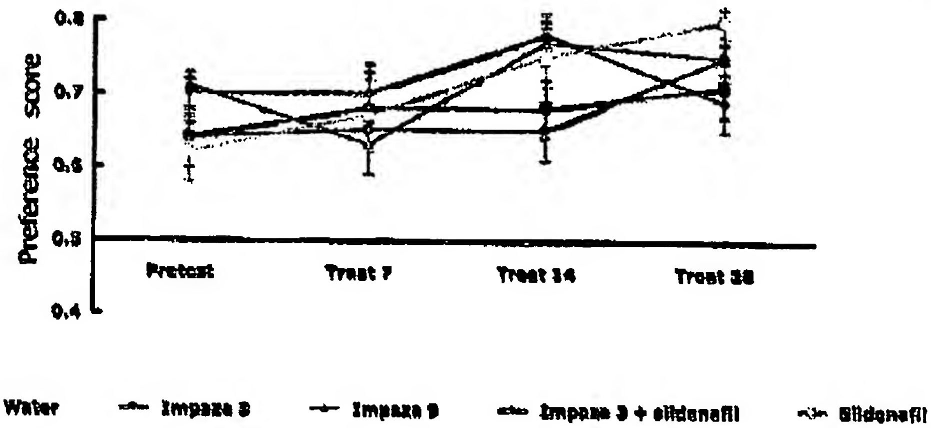

Figure 18. Mean ± S.E.M. preference score in the different treatments of male Wistar rats at the baseline and after 7, 14 and 28 days of treatment.

All groups had a preference score significantly above 0.5, meaning that they always spent more time in the vicinity of the sexually receptive female than in the vicinity of the male incentive (all Ps < 0.01).

When the difference in preference score between baseline and the tests performed at days 7, 14 and 28 of treatment was analyzed, a different picture emerged. There was a significant effect of treatment ($F(4,45) = 2.78$, $P < 0.05$) and of test ($F(2,90) = 9.38$, $P < 0.001$) and the interaction test x treatment was also significant ($F(8,90) = 2.55$, $P < 0.05$). The interaction prompted tests for simple effects of treatment for each test day. It turned out that there was a significant group difference only at the test performed on day 28 of treatment. When the effect of test was analyzed within each treatment, it turned out that the control group had a stable preference that did not change between tests. In all other groups, the effect of test was significant. The group treated with Impaza, 3 ml/kg, had a higher preference score on day 28 than it had at the baseline test. Impaza, 9 ml/kg, as well as Impaza, 3 ml/kg + sildenafil failed to alter the preference score. Sildenafil alone enhanced the preference score at the tests performed 14 and 28 days after the start of treatment. Data are illustrated in Fig. 19.

In order to further clarify potential treatment effects on sexual incentive motivation data obtained at the baseline test were compared to those obtained in the test performed on day 28 of treatment with the Wilcoxon test. A separate test was made for each treatment. It turned out that there was no change in preference score in the groups given water ($z = 1.48$, NS), Impaza 9 ml/kg ($z = 1.38$, NS) or Impaza, 3 ml/kg + sildenafil ($z = 0.46$, NS). The group given Impaza, 3 ml/kg as well as the one given sildenafil showed a significant increase in preference score between baseline and the test on day 28 of treatment ($z = 2.50$, $P < 0.05$ and $z = 2.80$, $P < 0.01$, respectively).

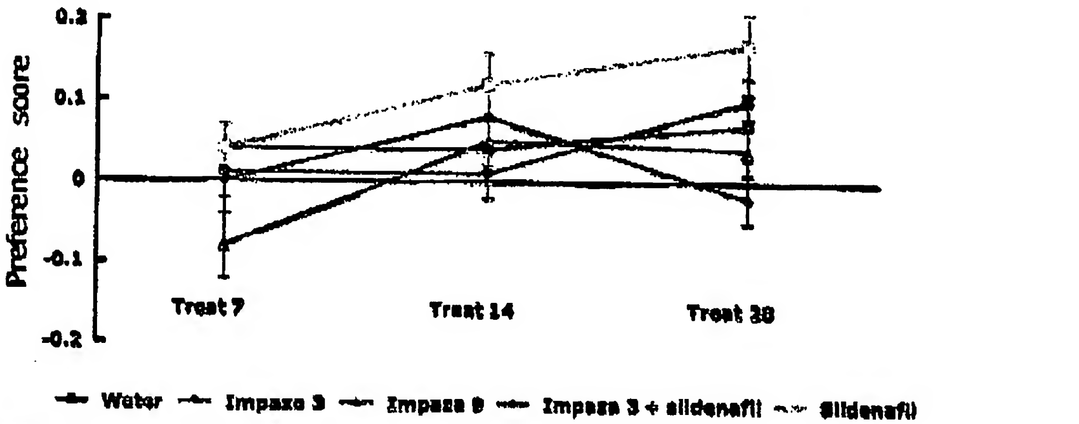

Figure 19. Mean ± S.E.M. of change in preference score from baseline in the different treatments of male Wistar rats.

2.2.1.2 Time spent with the receptive female vs. the intact male

When the time spent in the incentive zones (intact male and sexually receptive female) at the 4 tests (pretest and 3 tests during treatment) in the 5 groups of Wistar rats was evaluated with a three-factor mixed ANOVA there was no significant main effect of treatment ($F(4,45) = 1.51$, NS). There was an effect of test ($F(3,135) = 2.85$, $P < 0.05$) and of incentive (male vs. receptive female) ($F(1,45) = 195.08$, $P < 0.001$). There was no incentive x treatment ($F(4,45) = 0.72$, NS) or test x treatment ($F(12,135) = 0.87$, NS) interaction. To the contrary, the interaction test x incentive was significant ($F(3,135) = 9.73$, $P < 0.001$). The three-way interaction test x incentive x treatment was also significant ($F(12,135) = 2.56$, $P < 0.01$). For readability, the illustration of the data is made in two figures, one for the time spent in the male incentive zone (Fig. 19) and another for the time spent in the receptive female incentive zone (Fig. 20).

The interaction test x incentive is probably due to a small reduction in the time spent in the male incentive zone combined with a small increase in the time spent in the sexually receptive female incentive zone. The absence of interactions between test and treatment and incentive and treatment suggests that these effects are unrelated to the drug treatment. However, the three-way interaction test x incentive x treatment would suggest that some treatment had an effect at some test or tests and that this effect was specific to one of the incentives or of opposite direction for each incentive. Examining the data in Figs. 20 and 21, it appears that the sildenafil group progressively spent less time with the male and more time with the female. A similar trend, although less evident, can also be seen in the animals treated with Impaza, 3 ml/kg. Since there was no significant difference between treatments at any test, these proposals should be considered suggestive only. However, nonparametric tests reveal a slightly different picture. Impaza, 3 ml/kg, augmented the time spent in the female incentive zone between baseline and the test on day 28 of treatment ($z = 2.29$, $P < 0.05$) and reduced the time spent in the male incentive zone ($z = 2.50$, $P < 0.05$). Sildenafil had an identical effect ($z = 2.50$, $P < 0.05$, and ($z = 2.29$, $P < 0.05$, respectively). The other treatments were ineffective.

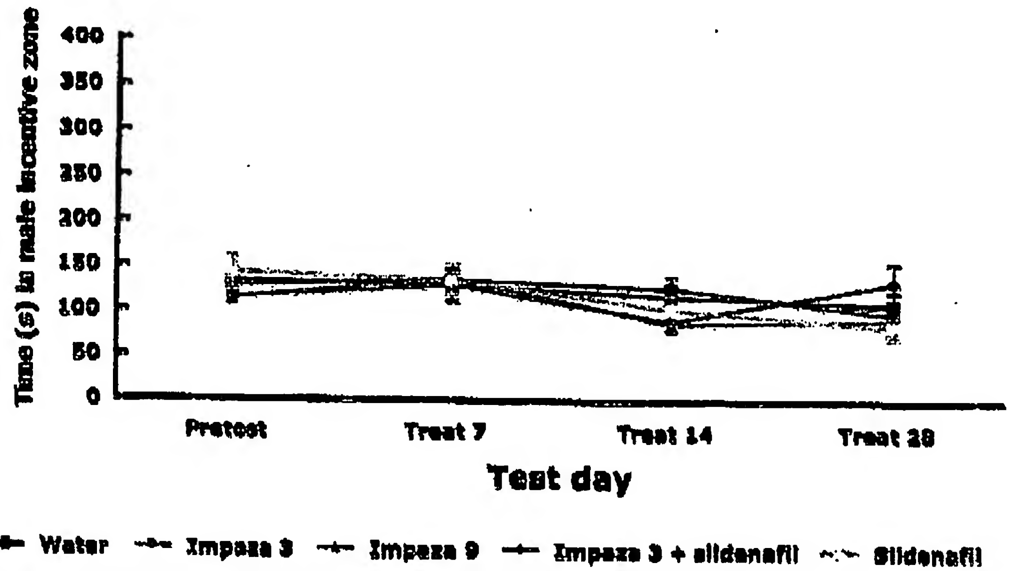

Figure 20. Mean ± S.E.M. time (sec) spent in the male incentive zone in the different treatments of male Wistar rats at the baseline and after 7, 14 and 28 days of treatment.

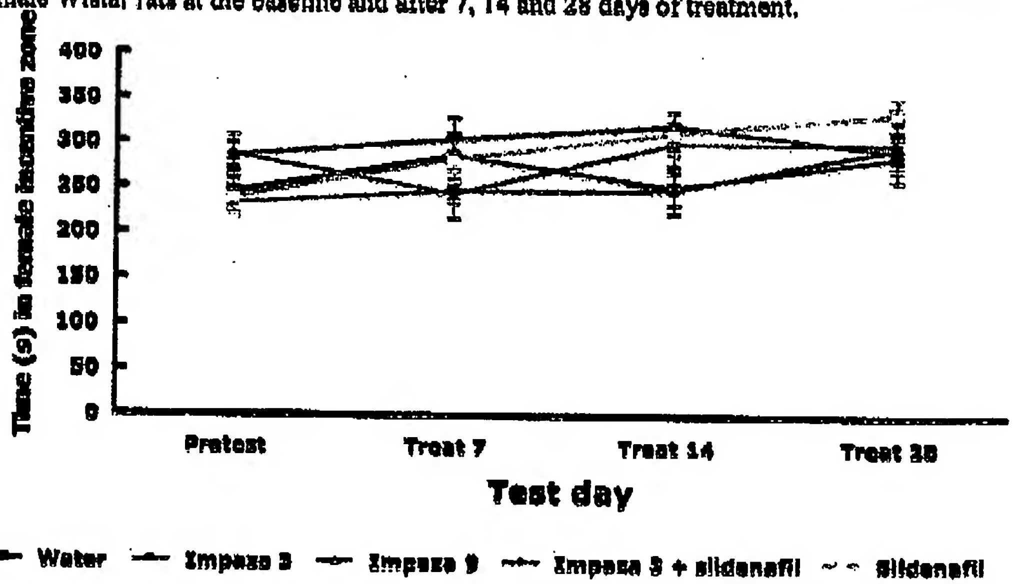

Figure 21. Mean ± S.E.M. time (sec) spent in the female incentive zone in the different treatments of male Wistar rats at the baseline and after 7, 14 and 28 days of treatment.

The analysis of the change from baseline in time spent in the incentive zones clarified the picture substantially. Both the test x incentive and treatment x incentive interactions were significant (F(2,90) = 3.38, $P < 0.01$ and F(4,45) = 3.16, $P < 0.05$, respectively). This confirms the proposal made above that the time spent with one incentive (the male) was reduced while that spent with the other incentive (the female) was increased. This is particularly the case for the animals treated with Impaza, 3 ml/kg and those given sildenafil alone.

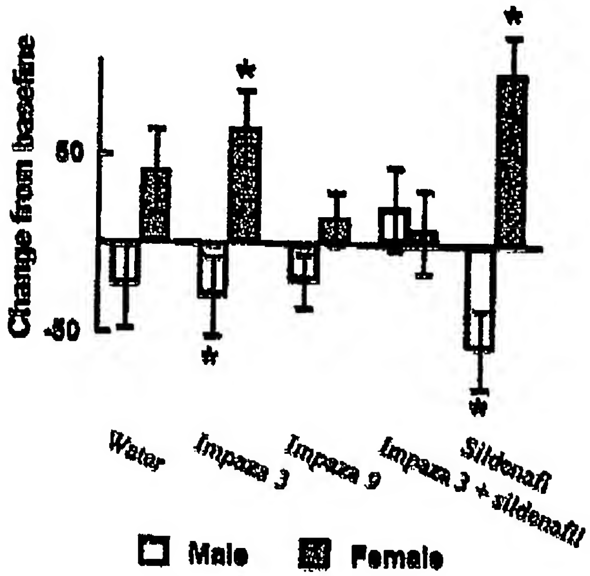

Figure 22. Mean ± S.E.M. change from baseline in time (sec) spent in the male and female incentive zones at day 28 of treatment. *, $P < 0.05$ (observed value compared to 0 (no change) with a *t*-test).

2.2.1.3 Number of visits to the incentive animals

Three-factor mixed ANOVA of the number of visits to the incentive animals at the 5 test occasions showed a main effect of test ($F(3,135) = 7.58$; $P < 0.001$). There was also an effect of incentive ($F(1,45) = 38.72$; $P < 0.001$) and of treatment ($F(4,45) = 3.04$, $P < 0.05$). The interactions test x treatment ($F(12,135) = 1.43$, NS), and incentive x treatment ($F(4,45) = 0.67$, NS) were nonsignificant. This was also the case for the interactions test x incentive and test x incentive x treatment ($F(3,135) = 1.89$, NS and $F(12,135) = 0.74$, NS, respectively). The effect of test appears to be due to a progressive decline in the number of visits with repeated testing, independently of treatment. This decline seems to be more evident with regard to the male incentive, but since the interaction test x incentive was nonsignificant, this is only an impression. The treatment effect is to be found in the sildenafil group. Post hoc comparisons established that

this group made fewer visits than the other groups, except the one treated with Impaza, 3 ml/kg, + sildenafil. The functional significance of this observation is unclear. The effect of incentive is clearly due to the fact that the subjects made more visits to the receptive female than to the male after all treatments at all tests. For readability, data are illustrated in two figures, one for the number of visits to the male incentive (Fig. 23) and one for visits to the female incentive (Fig. 24).

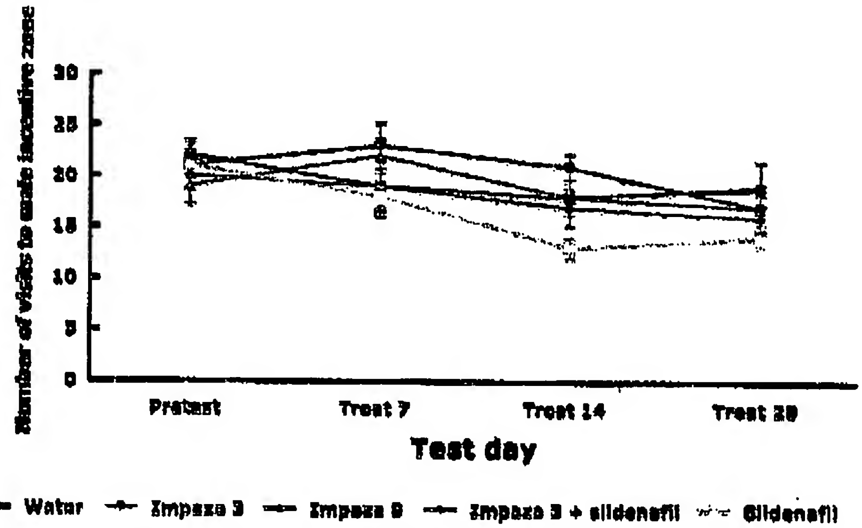

Figure 23. Mean ± S.E.M. number of visits to the male incentive.

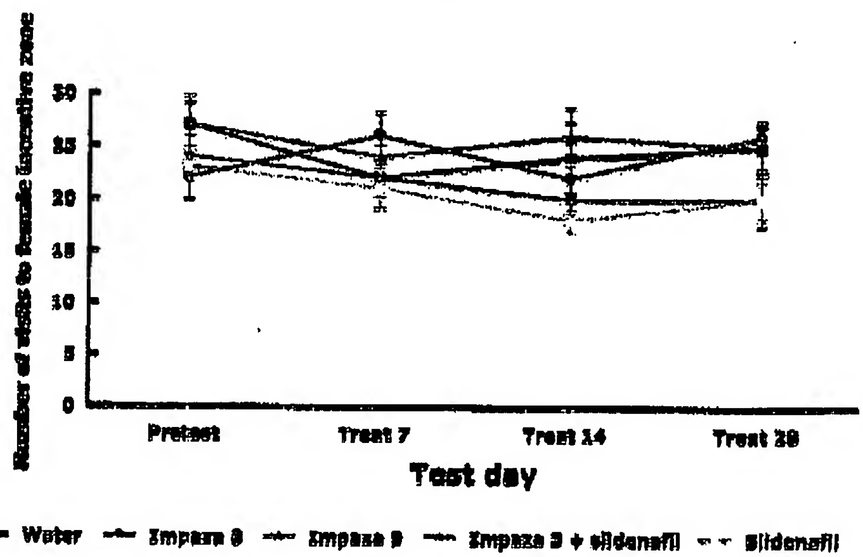

Figure 24. Mean ± S.E.M. number of visits to the female incentive.

2.2.1.4 Ambulatory activity

With regard to the distance moved during the test, two-factor ANOVA with test as within groups factor and treatment as between groups factor did not detect any difference between treatments ($F(4,45) = 0.83$, NS). There was a difference between tests, though ($F(3,135) = 19.48$, $P < 0.001$), but no interaction test x treatment ($F(12,135) = 1.08$, NS). These data show that the treatments did not affect a sensitive indicator of general activity. Activity declined with repeated testing, but this decline was independent of treatment. Data are found in Fig. 25.

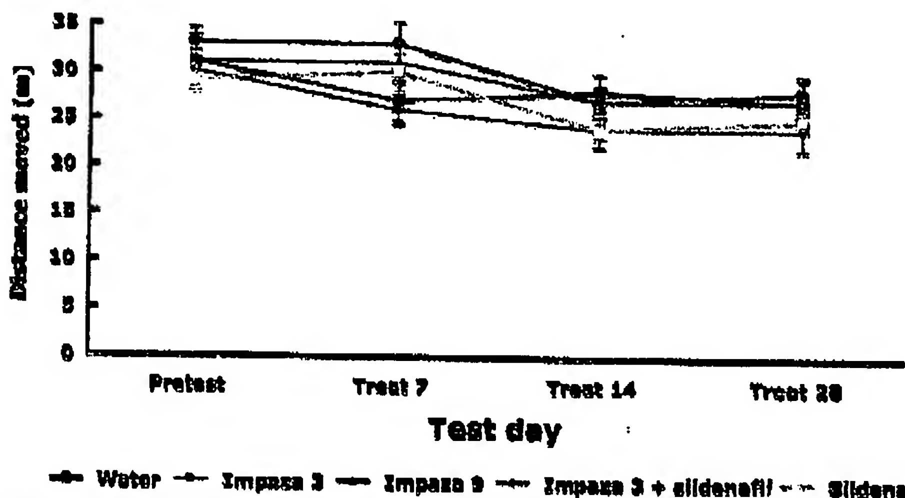

Figure 25. Ambulatory activity expressed as distance moved (in meters) during the sexual incentive motivation test in 5 groups of male Wistar rats at 4 tests.

Similar results were obtained when another indicator of motor function, the mean velocity of movement while moving, was analyzed. There was no effect of treatment, ($F(4,45) = 0.86$, NS) but there was an effect of test ($F(3,135) = 19.24$, $P < 0.001$). The interaction treatment x test turned out to be nonsignificant ($F(12,135) = 1.08$, NS). It is again concluded that there was no treatment effect on velocity of movement but there was a small reduction with repeated testing. Data are illustrated in Fig. 26.

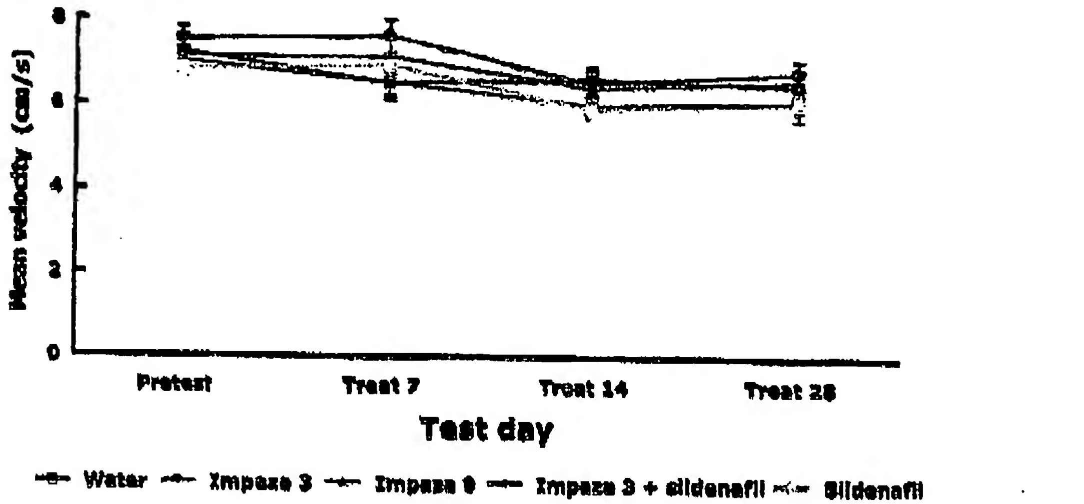

Figure 26. Ambulatory activity expressed as mean velocity of movement while moving (in cm/s) during the sexual incentive motivation test in 5 groups of male Wistar rats at 4 tests.

Finally, the time spent moving) was evaluated. As always, there was no difference between treatments ($F(4,45) = 1.03$, NS). The tests differed ($F(3,135) = 12.67$, $P < 0.001$) while the interaction treatment x test was nonsignificant ($F(12,135) = 1.18$, NS) Again, there was a small decline in the time spent moving with repeated testing, and this decline was unrelated to treatment. Data are illustrated in Fig. 27.

All indices of general activity coincides in showing an absence of treatment effect while there was a small reduction from the baseline test to the last treatment test.

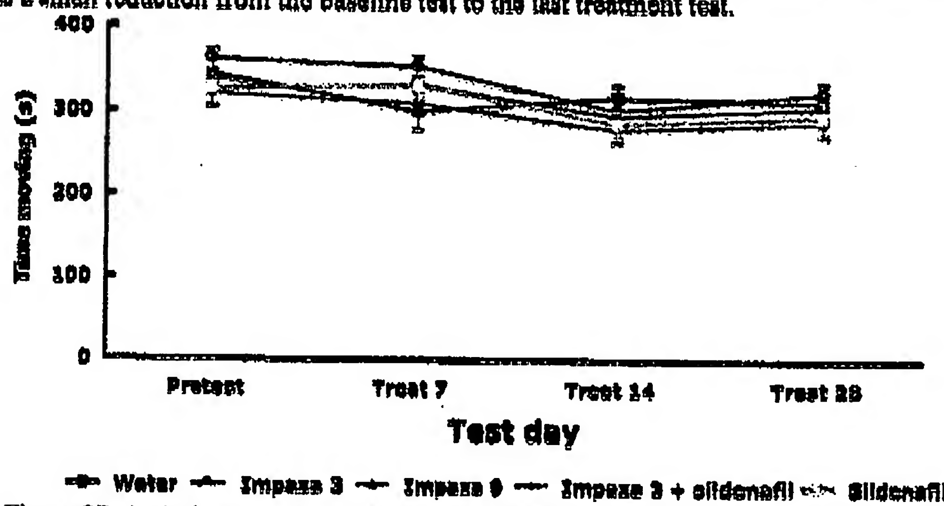

Figure 27. Ambulatory activity expressed as time not moving during the sexual incentive motivation test in 5 groups of male Wistar rats at 4 tests.

2.2.2 Copulatory behaviour

Chi-square tests revealed that the treatment groups did not differ with regard to the proportion of animals displaying at least one mount, intromission, or ejaculation at any test. There is no clear sign of any drug effect. However, if the proportion of animals mounting, intromitting and ejaculating within each treatment is compared over the 4 tests with Cochran's Q test, some interesting information is obtained. There is no change in the proportion of animals mounting. To the contrary, in the group treated sildenafil there was a significant change in the proportion of animals performing intromission ($Q = 8.05$, $P < 0.05$). Pairwise comparisons of treatments with the binomial test failed to confirm this, though. There was no effect in the other groups. The proportion of animals ejaculating within each group also changed in the animals treated with sildenafil ($Q = 10.71$, $P < 0.05$) and in the animals treated with Impaza, 9 ml/kg ($Q = 8.05$, $P < 0.05$). The binomial test showed that the only significant difference was between treatment days 7 and 28 in the sildenafil group. In the Impaza, 9 ml/kg, group the bionomial test failed to detect any significant difference. Since there was no difference between baseline and other test in any group, these data do not suggest a clear-cut drug effect, although the tendencies they reveal might be interesting. Data are illustrated in Figures 28 – 30.

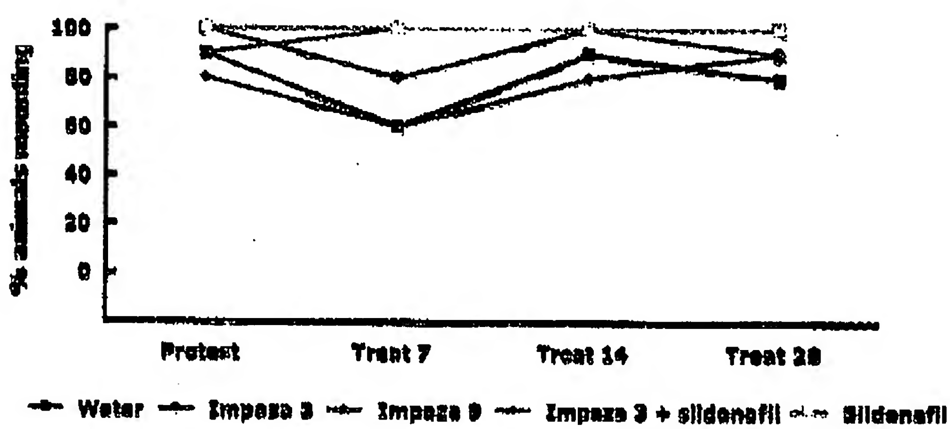

Figure 28. Proportion (expressed as percent) of male Wistar rats displaying at least one mount at the 4 tests.

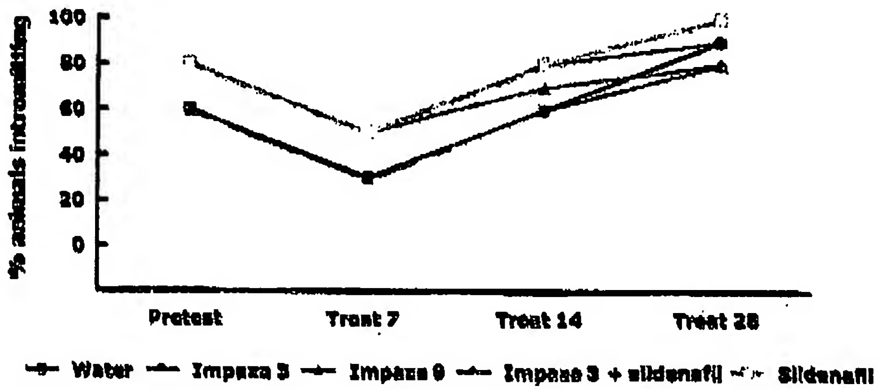

Figure 29. Proportion (expressed as percent) of Wistar rats displaying at least one intromission at the 4 tests.

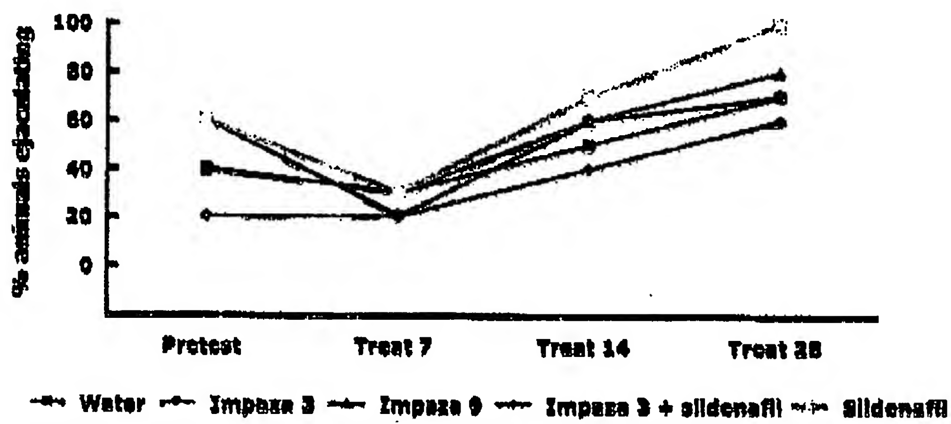

Figure 30. Proportion (expressed as percent) of Wistar displaying ejaculation at the 4 tests.

A two-factor mixed ANOVA of the number of mounts displayed in each treatment group at each test revealed that there was no significant difference between treatments ($F(4,45) = 0.13$, NS). Likewise, there was no effect of test ($F(3,135) = 0.97$, NS) and no interaction treatment x test ($F(12,135) = 0.70$, NS). Data are illustrated in Fig. 31.

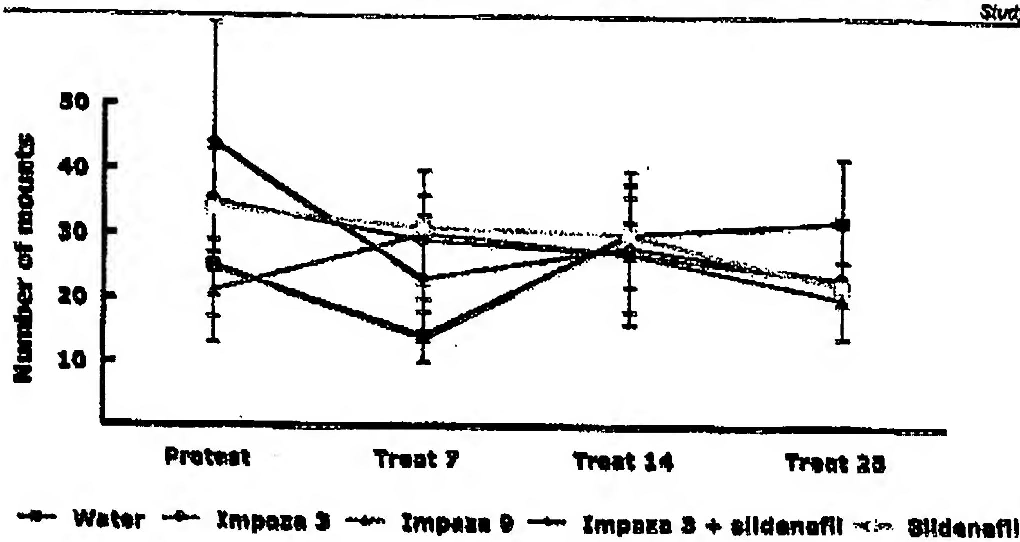

Figure 31. Number of mounts (mean ± S.E.M.) displayed by the Wistar rats at baseline and after 7, 14 and 28 days of treatment.

When a similar analysis was performed for the number of intromissions, it turned out that there was no treatment effect ($F(4,45) = 1.43$, NS). To the contrary, there was a difference between tests ($F(3,135) = 7.93$, $P < 0.001$) but no interaction treatment x test ($F(3,135) = 0.46$, NS). As can be seen in Fig. 32, the number of intromissions first showed a reduction below baseline at day 7, and then an increase at days 14 and 28. *Post hoc* test showed that there was no significant difference between baseline and any test, while the test performed on day 7 of treatment differed from the tests performed at days 14 and 28. These differences were unrelated to the treatments, and are of little interest.

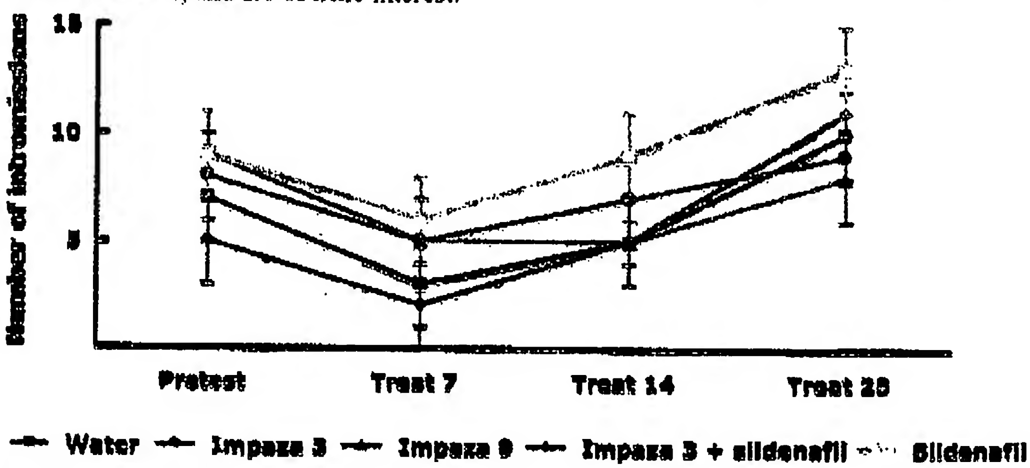

Figure 32. Number of intromissions (mean ± S.E.M.) displayed by the Wistar rats at baseline and after 7, 14 and 28 days of treatment.

As was the case with the Fisher 344 rats, the other measures of sexual behaviour could not be obtained from every animal at every test. As mentioned, repeated measures analyses of these data are, therefore, useless. Instead, a separate analysis was performed for each test day. The treatments were compared with one-factor ANOVA. At baseline, there was no group difference at all (all $Ps > 0.13$). This was also the case for the tests performed at days 7, 14 and 28 of treatment ($Ps > 0.14$ at day 7, > 0.11 at day 14 and > 0.39 at day 28). Thus, none of the treatments affected any parameter of copulatory behaviour. Data from the test at day 28 of treatment are shown in Table 9.

Table 9. Copulatory behaviour in Wistar males at the test performed on day 28 of treatment. Data are mean ± SEM.

Behaviour parameter	Treatment				
	Water	Impaza 3	Impaza 9	Impaza 3 + sildenafil	Sildenafil
Mount latency	67 ± 32	59 ± 27	25 ± 7	41 ± 8	25 ± 8
Intromission latency	92 ± 32	74 ± 28	31 ± 11	110 ± 55	51 ± 19
Ejaculation latency	641 ± 97	468 ± 105	423 ± 83	440 ± 111	591 ± 122
Postej. interval	304 ± 19	264 ± 12	276 ± 31	280 ± 19	304 ± 19
N of mounts	32 ± 10	23 ± 9	20 ± 6	23 ± 9	22 ± 5
N of intromissions	10 ± 2	9 ± 1	8 ± 2	11 ± 2	13 ± 2
Intromission ratio	0.35 ± 0.1	0.38 ± 0.06	0.39 ± 0.01	0.38 ± 0.08	0.45 ± 0.07

The data reported in Table 9 suggest that no treatment enhanced erectile capacity.

2.2.3 Penis length

Measurements of the length of the erect penis during mount or following withdrawal after an intromission or ejaculation failed to establish any effect of treatment. Data from the test performed on day 28 of treatment are shown in Table 10. Changes from baseline were also nonsignificant (data not shown). Nonparametric tests showed an occasional significance, but it does not seem to be indicative of any systematic treatment effect on penis length.

Table 10. Penis length in Wistar males at the test performed on day 28 of treatment. Data are mean ± SEM.

Behaviour	Treatment				
	Water	Impaza 3	Impaza 9	Impaza 3 + sildenafil	Sildenafil
Mount	0.35 ± 0.02	0.34 ± 0.02	0.33 ± 0.01	0.32 ± 0.01	0.34 ± 0.01
Intromission	0.42 ± 0.03	0.37 ± 0.02	0.39 ± 0.01	0.37 ± 0.03	0.40 ± 0.02
Ejaculation	0.48 ± 0.04	0.47 ± 0.06	0.46 ± 0.05	0.50 ± 0.07	0.43 ± 0.05

Notes: Length is expressed in arbitrary units (mm on the projection screen).

Discussion

1. Comparisons between strains

Fisher 344 males approached the sexually receptive female more intensely than the Wistar males. This difference appeared stable, and persisted throughout the 4 tests. Since the factors determining the intensity of approach behaviours are badly known, it is not possible to propose any informed explanation for this difference. Copulatory behaviour was essentially similar in the two strains, and at the end of the experiment there were no differences of importance. This is also the case for penis length at mount, intromission and ejaculation. In view of the similarities between the two strains it appears difficult to attribute any differential drug action to differences in sexual behaviours.

There are no earlier studies in which the Wistar and Fisher 344 strains have been compared with regard to copulatory behaviour or sexual motivation. This means that we do not even know if the differences observed here are specific to the animals employed in this particular experiment, or if they are a stable characteristic of these strains.

2. Treatment effects in Wistar rats

The data from the sexual motivation test show that none of the treatments affected any indicator of general activity, viz. distance moved, speed of movement or time spent moving. To the contrary, Impaza, 3 ml/kg and sildenafil showed a clear tendency to increase sexual motivation. This effect became particularly apparent when change from baseline was analyzed. Curiously enough, when sildenafil was given together with Impaza, 3 ml/kg, there was no effect, and the larger dose of Impaza, 9 ml/kg, was also ineffective. This is not easy to explain, but it certainly suggests that there is an optimal level of NO synthesis for sexual incentive motivation. Stimulation beyond that level may activate systems interfering with its expression.

Copulatory behaviour was not reliably modified by any treatment in the Wistar strain, although some nonsignificant tendencies for facilitation were evident. This concerns both the

number of intromissions and the proportion of animals ejaculating after Impaza, 9 ml/kg and sildenafil. The intromission ratio, supposedly an indicator or erectile capacity, was not affected. Similarly, no significant effect on penis length could be found. However, there was a tendency towards increase after Impaza, 9 ml/kg and sildenafil.

Concerning sildenafil, there is limited evidence that the drug may facilitate some aspects of male rat copulatory behaviour (Ferrari et al., 2002; Giuliani et al., 2002; Ottani et al., 2002). This notion is partly reinforced by present data, although the effects found here rarely are statistically reliable. To the contrary, the effects on sexual motivation found in the present study appear to be more solid. However, there are no clinical reports of enhanced sexual motivation following treatment with sildenafil, despite the quite extensive use of the drug. One reason may be that clinical studies have focused on evaluations of erectile function rather than on motivation. Further studies, both clinical and preclinical, might confirm the present observations.

3. Treatment effects in Fisher 344 rats

No effect was observed on sexual motivation regardless of how data were analyzed. This is in contrast to an earlier study in which old Fisher 344 rats showed enhanced motivation after treatment with Impaza and sildenafil (Chu and Ågmo, 2007). One reason might be that untreated old Fisher 344 rats fail to show any sexual motivation at all, whereas the young but adult Fisher rats used in the present experiment actually had a higher initial level of motivation than the Wistar rats. Perhaps that NO synthase activity was already at an optimal level in these rats, making further increases inefficient.

Both sildenafil and Impaza, 9 ml/kg, enhanced the intromission ratio at day 28 of treatment. It must be observed that the enhanced intromission ratio was not associated with any systematic, significant change in observable penis length. However, there was a tendency for penis length to be enhanced after treatment with Impaza, 9 ml/kg or sildenafil. The fact that the ANOVA failed to reveal a significant effect may be because penis length measurements are not sufficiently sensitive or that it is the degree of erection immediately preceding penile insertion (intromission) which is the crucial determinant of whether a mount will end in intromission or not. It must be remembered that penis length measurements were made during pelvic thrusting at mounts that never succeed in penetration while it was made immediately after withdrawal if penetration occurred. It was not possible to measure penis length immediately before penetration since this is an extremely fast event. Furthermore, vaginal penetration is associated with contraction of the ischiocavernosus muscles (Holmes et al., 1991), enhancing intracavernous pressure above systolic blood pressure (Bernabé et al., 1999). This means that erection associated with penetration in rats is not only a vascular but also a somatic response. The activity of the ischiocavernosus muscles is reduced upon withdrawal, suggesting that the remaining erection is mainly a vascular response. This was the reason why it was considered most informative to quantify erection upon withdrawal. On the other hand it can be maintained that the measurement of penis length must give some useful information, since a clear difference usually was found between mount and intromission – ejaculation. It is known that intracavernous pressure is higher at intromission than at mount, and higher at ejaculation than at intromission (Giuliano et al., 1994), and part of these increases in pressure are considered to be vascular. Thus, there appears to exist some relationship between intensity of erection and measurable penis length.

4. Strain differences in treatment effect

The difference between Fisher 344 and Wistar rats with regard to treatment effects on sexual motivation as well as the difference with regard to effects on intromission ratio is difficult to explain. Several studies have reported differences between Fisher 344 and Wistar rats in several behavioural tests. For example, Fisher 344 rats appear to be more fearful than Wistar rats in tests for anxiety and respond with larger serotonin release to a stressful situation (Rex et al., 1999). Likewise, strain differences in several neurotransmitter concentrations and affinities as well as differential sensitivity to morphine have been reported (Sudakov et al., 1993). However, it is unclear whether any of these behavioural and neurochemical differences can explain present results. Until more data has been accumulated, any speculation as to exact causes for the strain differences observed would be premature.

A somewhat speculative proposal for explaining the differences between Wistar and Fisher 344 rats can be based on the observation that several of the P450 cytochromes are far more responsive to induction by xenobiotics in the Fisher 344 than in Wistar strain. It also appears to exist a baseline difference between the two strains (Larsen et al., 1994). It has been proposed that P450 may be involved in NO synthesis (Keseru et al., 2000), and recently it has even been reported to be of importance for erection (Jin et al., 2006). This reasoning could easily explain the more evident effect of Impaza on erectile mechanisms in the Fisher 344 rats, assuming that endothelial NO synthase somehow interacts with the P450 system.

CONCLUSIONS

The findings of the present study suggest that a low dose of Impaza (3 ml/kg) and sildenafil, 3 mg/kg twice weekly, enhance sexual motivation and perhaps also some aspects of copulatory behaviour in Wistar rats. In Fisher 344 rats, the larger dose of Impaza, 9 ml/kg, and sildenafil appear to facilitate vaginal penetration through enhanced erection. No other effect could be found in Fisher 344 rats. In sum, Impaza appears to have prosexual effects to a larger extent than sildenafil.

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Date: November 26, 2007

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PRECLINICAL STUDY REPORT

Sexual behavior and erectile function in old rats: the influence of 4-week treatment

Study Director:

Anders Ågmo, Professor
Institute for Psychology, University of Tromsoe, Norway

Study Sponsor:

"Materia Medica Holding" company, Moscow, Russia

First version: 27 November 2006

Final version: 08 December 2006

Main objective

Evaluate the efficacy of the tested drug (provided by "Materia Medica Holding" company, Russia) in an animal model of erectile/ sexual dysfunction.

Test substance:
Antibodies to C-terminal fragment of endothelial NO synthase (20 amino acids), ultra-low doses for oral administration (mixture of homeopathic dilutions C12, C30, and C200). The tested substance is an active ingredient of a therapeutic approved in Russia for the treatment of erectile dysfunction (impaza).

Reference substance:
Sildenafil citrate (selective inhibitor of phosphodiestherase type 5, a standard therapy for erectile dysfunction in humans).

STUDY DIRECTOR'S AUTHENTICATION

I, the undersigned, hereby declare that the work described in this report was performed under my supervision as Study Director and that the final report provides a true and accurate record of the results obtained.

Anders Agmo,
Professor of biological psychology
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STUDY ORGANISATION

Study Sponsor

The experiments are sponsored by OOO NPF "Materia Medica Holding", a company organised and existing under the laws of Russian Federation.

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3-rd Samotyochnyi per. 9,
127473, Moscow,
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Phone/fax: +7 495 631 24 76 (Research and Development department)
Project manager: Andrey Martyushev-Poklad, M.D., Ph.D.

Test facility

The experiments were carried out in the animal facilities of the Institute of Medical Biology, Faculty of Medicine, University of Tromsoe.

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Personnel

Study Director: Anders Ågmo, Ph.D., Professor.
Work done by: Xi Chu, graduate student.
Animal care and some other assistance: Ragnhild Osnes and Stig Rune Olsen, laboratory technicians.
Date for start of experimental work: 18.04.2006.
Date for completion of experimental work: 12.07.2006.

Archiving

The raw data are kept by Dr. Anders Ågmo at the University of Tromsoe.

Schedule

Numbers refer to weeks of 2006.

Weeks 16 – 23. Acquisition of copulatory experience, familiarization to sexual incentive motivation test environment.

Week 24 – 28. Drug treatment started on June 12 (one third of the animals), June 13 (another third) and June 14 (the last third) and ended on July 10, 11 and 12, respectively.

MATERIALS AND METHODS

Test subjects

1) A total of 50 experimentally and drug naive, 18 months old male Fisher 344 rats (NIA, Bethesda, MD) were used. At the start of treatment the age of males amounted to 19.5 months, average weight – to 457.5±34.2 g (range 390-530 g).
2) Eight male rats were used as neutral incentives in the sexual incentive motivation part of the experiment. These males (300 – 400 g) were of the Wistar strain and bought from B&K, Sollentuna, Sweden.
3) Sixteen females (300-350 g, B&K, Sollentuna, Sweden) were used as copulation partners. They were ovariectomized under isoflurane anesthesia at least 2 weeks before use and given estradiol benzoate (25 µg, Sigma) 48 hrs before testing and progesterone, 1 mg, about 4 hrs before each session.
The rats were housed in pairs in Macrolon IV cages, in a temperature controlled animal room at +21°C ± 1 °C, at a relative humidity of 55% ± 10% and on a reversed 12 h light/dark cycle (lights on 23:00 -11:00), with free access to water and food. Standard certified dry pelleted food, rodent low protein, supplied by B&K Universal, Sollentuna, Sweden was used. Tap water was available to the animals ad libitum in Macrolon bottles. The water was checked daily and bottles changed twice a week.
All experimentation was approved by the local laboratory animal care and experimentation committee. The animals were housed according to the rules of European Convention (EC, 1990) and to the rules of National Research Council (NRC, 1996) USA.

Test articles

Hormones used for the induction of sexual receptivity in the females
Crystalline β-estradiol (Sigma, batch 88H3787) and progesterone (Sigma, batch 89H0640) were mixed with peanut oil (Apotekproduksjon, lot 4E090/1) and heated to 60 °C for 24 hrs in order to produce a stock solution. This was diluted in peanut oil to the appropriate concentration (125 µg/ml for estradiol benzoate and 5 mg/ml for progesterone). The steroids were injected s.c. in a volume of 0.2 ml/rat.

Experimental drugs
Test substances:
1) Antibodies to C-terminal fragment of endothelial NO synthase (20 amino acids), ultra-low doses for oral administration (anti-NOS) – active ingredient of impaza (a therapeutic approved in Russia for the treatment of erectile dysfunction). Two technological versions of the drug substance were presented in Sample 1 (version currently used in manufacturing) and Sample 2 (experimental version).
Two different samples (batches) of anti-NOS were provided as water solution ready for use (no smell, no taste) in 250 ml plastic vials, delivered via DHL (arrived on April 28, 2006) and given by gavage once daily (at 9-10 a.m.) for 28 days. On the days of tests, anti-NOS was given 1-2 hours before the start of testing.
Sample 1 of anti-NOS was administered in 2 doses: 1 ml/rat (10 rats) and 3 ml/rat (10 rats).
Sample 2 of anti-NOS was administered in 1 dose: 1 ml/rat (10 rats).

2) Passive control:

vehicle (distilled water provided by the Physiology Department, University of Tromsø) was given by gavage, 1 ml/rat daily for 28 days (10 rats). On the days of tests, vehicle was given 1-2 hours before the start of testing.
3) Active control (was provided by the Sponsor):
sildenafil citrate was given 3 mg/kg p.o. twice weekly for 4 weeks (10 rats). On the days of tests, sildenafil was given 1-2 hours before testing in average volume of 1.37±0.13 ml.
One tablet of sildenafil citrate 25 mg (Viagra, Pfizer, USA) was thoroughly crushed and dissolved in saline solution (made by adding 9 g of sodium chloride to 1 l of distilled water) on the basis of 1,5 mg in 1 ml (about 17 ml).
Batch numbers of sildenafil: 4104056; 5108959.

Methods

As an OECD Test Guideline is not available for the present study, the following protocol has been chosen as the Guideline:
Ågmo, A. (1997). Male rat sexual behavior. Brain Research Protocols, 1(2): 203-209.
The procedures employed here are standard techniques used for analyses of copulatory behavior and sexual motivation (defined as the urge to seek contact with an individual of the opposite sex). There are many minor variations, such as size and shape of the observation arena, duration of the test, etc.. However, none of these variations have any systematic effect on the behavior observed. The capacity to achieve vaginal penetration during the test for copulatory behavior has been found to be exquisitely dependent on appropriate erection, and constitutes the most sensitive system for evaluating the efficiency of proerectile compounds *in copula*.

Sexual incentive motivation test environment

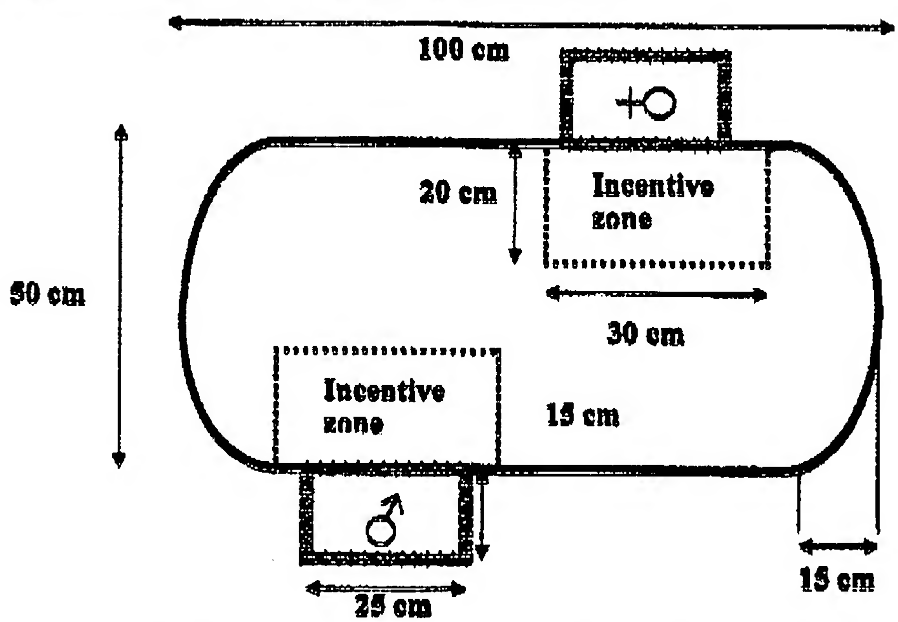

Fig. 1. The apparatus for evaluating sexual incentive motivation. For further details, see text

The test for sexual incentive motivation reveals subtle changes in general arousal, expressed as forward locomotion and speed of movement, in addition to changes in sexual interest. The observation arena is illustrated in Fig. 1. The arena walls and the incentive animals cages were made of sheet steel covered with a black plastic surface. Dark grey polyvinylchloride was used for the floor. The incentive animal cage wall facing the arena was of a 1 x 1 cm stainless steel wire mesh. The apparatus was located in a room adjacent to the animals' room. A video camera was installed above the arena. The camera was connected to a computer. The experimental subject's position was determined online with a videotrack system (Ethovision, Noldus, Wageningen, The Netherlands). An incandescent light bulb provided dim white light (about 5 lux in the arena).

Copulatory behavior test environment

Black sheet-steel cages (40 x 60 x 40 cm high) with Plexiglas front and glass floor were positioned over a mirror inclined 45 degrees. This allowed for a simultaneous side and ventral view of the copulating male. Tests were recorded on videotape with a 2-camera system connected to a VCR via a multiplexer.

Detailed description of procedures

Habituation of the male rats to sexual incentive motivation tests

The animals were familiarized to the observation arena during 3 sessions of 10 min each. During these sessions, incentive animal cages were empty.

Sexual incentive motivation tests

Before each experimental session the arena and the incentive animal cages were carefully washed with a 0.1 % acetic acid solution. The incentive animals were then placed in their respective cages. About 5 min later the first experimental subject was introduced into the middle of the arena. Immediately thereafter, the experimenter left the room and did not return until just after the end of the 10 min observation period. The subject was then gently removed from the arena, and the following rat was immediately introduced. No cleaning was performed between trials within a session. The position of the incentive animals were semi-randomly changed throughout the experimental session. At the end of every session, half of the animals had had the incentives in one position and the other half in the other. Care was taken to avoid that any single animal had the incentive animals in the same position in more than two consecutive sessions. Spatial location was, therefore, a useless predictor of the state of the incentives. In all experiments, the incentives were a receptive female (Wistar, about 6 months old at the beginning of experiments) and an intact male (Wistar, about 6 months old at the beginning of the experiment). The receptive female had always received the hormone treatment mentioned previously. All incentive animals were sexually inexperienced. For more details of procedure, see Ågmo, 2003, Ågmo et al., 2004.

Tests for copulatory behavior

Copulatory behavior was observed in a room separate from the sexual incentive motivation test. To assure that contextual conditioning during copulation could not affect tests for sexual incentive motivation, the copulation test room differed from the incentive motivation test room in several ways. It was brightly lit (about 300 lux in the observation cages), the furniture was different and the general arrangement of the room was also different. For example, the

observation cages were located on a table whereas the incentive motivation test arenas were located on the floor.

The male was put into the observation cage about 5 min before a receptive female was introduced. Copulatory behavior was then observed until the 1st ejaculation. The following behavioral parameters were recorded with in-house software: Mount latency (time from introduction of the female until the first mount with pelvic thrusting), intromission latency (time from introduction of the female until the first mount with vaginal penetration), ejaculation latency (time from the 1st intromission until ejaculation), the postejaculatory interval (time between the ejaculation and the next intromission), number of mounts, and number of intromissions. The intromission ratio (number of intromissions / (number of mounts + number of intromissions) were also calculated. This is the most sensitive, behavioral measure of erectile functioning. If no mounting occurred, the test was terminated after 15 min. It was also terminated if the ejaculation latency became > 30 min or the postejaculatory interval longer than 15 min. A more extensive description can be found in: Ågmo, 1997.

In addition, erect penis length during mount and/or following withdrawal after intromission were estimated from the video record. This estimation was not possible at every mount or intromission because of an unsatisfactory view, but we estimated that about half of the copulatory events provided acceptable video images. The amount of data obtained in this way was not enough for statistical analysis.

Design

The following five groups of 10 rats each were employed:

Group 1 - 3 for the investigational drug in doses indicated above (*Experimental drugs*). Daily oral administration (gavage).

Group 4 Vehicle (control). Daily oral administration.

Group 5 Sildenafil, 3 mg/kg p.o. Twice weekly. The sildenafil dose of 3 mg/kg p.o. was intermediate between doses that earlier had been found effective on male rat sexual behavior (Ferrari et al., 2002; Giuliani et al., 2002; Ottani et al., 2002). It was far above the dose needed to potentiate the effects of apomorphine on intracavernous pressure (0.1 mg/kg; Andersson et al., 1999). However, that study had employed intravenous administration and was, therefore, not directly comparable.

On days 1, 2, 4, 5 and 6 each week the rats of sildenafil group received vehicle (so that the animals were exposed to a similar amount of handling as in other groups); on days 3 and 7 of each week they received sildenafil, 3 mg/kg. On day 7 (the day of tests), the drug was given 1-2 hours before testing.

Behavioral testing was performed on days 0 (baseline test) and at treatment days 7, 14 and 28. On test days, the compounds were administered 1 h before observation.

Prior to the baseline test, the subjects acquired sexual experience at several pre-experimental tests. After the last behavioral tests, animals were euthanized and penile tissue immediately removed, frozen in liquid nitrogen and stored at −70 °C for future analyses.

Records

The following primary records (raw data) were made in the course of the study:

1) Experimental register (journal/ log) describing all procedures and manipulations performed with animals in the course of the study (day by day) (*photocopies of paper originals sent to Sponsor*).
2) Videotapes for tests for copulatory behavior – for all animals were made and provided to the Sponsor as raw data; the coordinates of the experimental rats' position in the incentive motivation test environment, recorded with a frequency of 5 Hz, are stored on the lab computer's hard disk, and can be made available at any moment.
3) Transcripts for all videotapes with all parameters mentioned above for each rat (*electronic format*)
4) Lists of all parameters derived from p.3 in the form of electronic tables designed for data processing and statistics (*electronic format*).

The records mentioned in 1) and 2) were provided to the Sponsor by mail; electronic records mentioned in 3) and 4) were sent to the Sponsor by e-mail.
The following raw data are stored by the University: The electronic files generated by the video track system; the electronic files generated by the copulatory behavior observation program.
Two originals of Study Reports are sent to the Sponsor, one original of Study report is stored by the University.

Data processing and statistics

Sexual motivation was quantified in several ways. Most important for evaluating changes in the sexual incentive value of the receptive female are the *preference score* (time spent in the female incentive zone/(time spent in the female incentive zone + time spent in the male incentive zone)) and *time spent in the female incentive zone*. There need to be a statistically significant change on both parameters if an effect on sexual motivation is to be considered. A double criterion is needed in order to avoid false positive effects. An increased preference score may be a result of either increased time in the female zone or reduced time in the male zone or a combination of both. However, reduced time in the male zone without a concomitant increase in time in the female zone does not necessarily indicate enhanced sexual incentive motivation. At the same time, an increase in the time spent in the female zone could be a consequence of increased attractivity of any incentive animal and is therefore not a sufficient indicator of increased sexual incentive motivation. Similar arguments could be made for reduced sexual incentive motivation. The use of both criteria (change in preference score *and* a corresponding change in time spent in the female zone) avoids the pitfalls of them when used singly. The preference score was analysed with two-factor ANOVA with repeated measures on one factor, the between-groups factor being treatment and the within-groups factor being test. For a more detailed analysis of the results obtained at day 28 of treatment, an analysis of covariance of the preference score was performed with treatment as factor and pretest preference score as covariate. In addition, the nonparametric Mann-Whitney U-test was employed for comparing each treatment with water. The time spent in the incentive zones was evaluated by three-factor ANOVA with repeated measures on two factors, the within group factors being incentive (male, female) and test and the between group factor being treatment. Data from day 28 of treatment were evaluated with an analysis of covariance with treatment as between-groups factor, incentive as within-groups factor and pretest time spent in the male and female zones as covariates. Because of significant interaction, tests for simple main effects within treatment were performed. Indices of ambulatory activity at all tests were analysed as the preference score, while the number of visits to the incentives were analysed like the time spent in the incentive zones.

Data from the copulatory behavior tests were limited to the proportion of subjects displaying mount, intromission or ejaculation. Treatments were compared with the chi-square test.

Deviations from the study protocol

Most unfortunately, one animal in the group to be treated with Sample 1, 1 ml, died before beginning of drug administration. The same occurred to a rat in the group to be given Sample 1, 3 ml. The local veterinarian performed autopsy but failed to identify the causes of death. It was attributed to old age. Two other animals lost weight and showed signs of bad health before the last drug-treatment test. One belonged to the group given Sample 1, 1 ml, and another to the group given sildenafil. These animals were eliminated from all analyses.
A software failure in the copulatory behavior recording program corrupted the data file corresponding to one rat in the group given Sample 1, 1 ml. It was necessary to reconstruct the record from the raw computer file. This animal did not display any copulatory behavior in any test. Thus, no data were lost despite the software failure.
The videotracking program gave incorrect data for one rat (number 6) in the group treated with Sample 1, 1 ml, with regard to the number of visits to the female incentive zone at pretest and for rat 23 in the group given water at the test on day 14 of treatment. This was due to the fact that the almost immobile rat moved its point of gravity back and forth over the line delimiting the incentive zone. This was corrected by imposing a minimum movement distance (5 cm) in the program. Thereby, the number of visits for rat number 6 changed from 146 to 18, and that of rat number 23 changed from 136 to 16.

Results

1.1 Sexual incentive motivation

1.1.1 Preference score

The preference score obtained at the 4 tests (pretest and 3 tests during treatment) in the 5 groups is illustrated in Fig. 2. Data were evaluated with a two-factor mixed ANOVA with treatment as the between groups factor and test as within groups factor. There was no significant main effect of treatment ($F(4,41) = 2.12$, NS) or of test ($F(3,123) = 1.20$, NS) and there was no interaction treatment x test ($F(12,123) = 1.34$, NS). Data are shown in Figure 2.

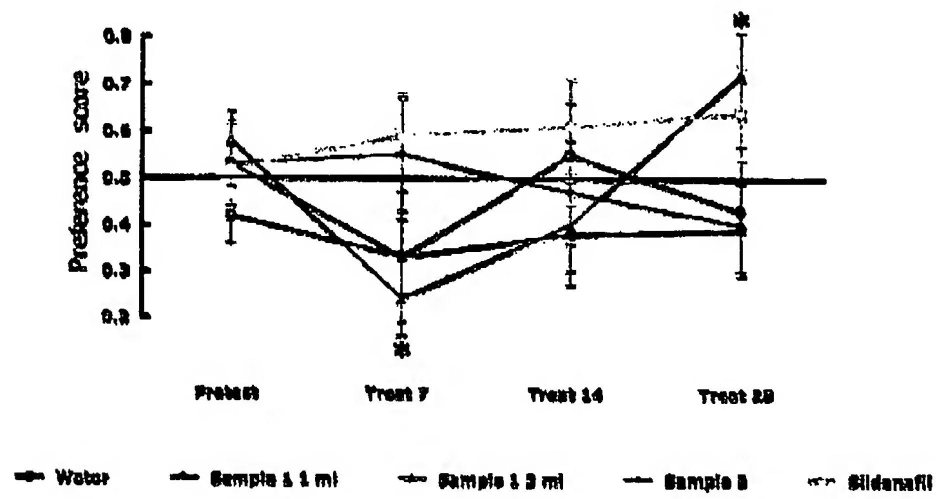

Figure 2. Mean ± S.E.M. preference score in 5 groups of male rats at 4 tests.

When the preference score was compared to chance, that is a score of 0.5, it was found that the group treated with Sample 1, 3 ml, had a preference score significantly below 0.5 at the test on day 7 of treatment ($t(8) = 3.36$, $P = 0.01$) while it was significantly above 0.5 at the test on day 28 of treatment ($t(8) = 2.51$, $P < 0.05$). None of the other treatments produced a score that differed from no preference at any test.

A detailed analysis of the preference score obtained at day 28 of treatment was then performed. An analysis of covariance with the preference score obtained at pretest as covariate revealed that the treatments differed ($F(4,40) = 2.98$, $P < 0.05$) . Further analysis showed that Sample 1, 3 ml, differed from water. No other difference was obtained. In order to substantiate this result, the preference score obtained in each treatment group was compared to water with the Mann-Whitney U-test. Again, the animals treated with Sample 1, 3 ml, differed from water (Mann-Whitney's $U = 16.00$, $P < 0.05$) while none of the other treatments did. Data from the test at treatment day 28 are illustrated in Fig. 3.

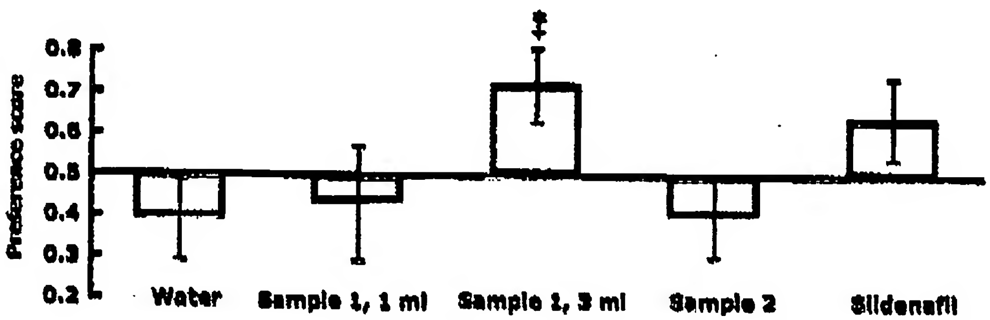

Figure 3. Mean ± S.E.M. preference score in 5 groups of male rats at the test on day 28 of treatment. *, different from no preference, a score of 0.5, $P < 0.05$; +, different from water, $P < 0.05$.

1.1.2 Time spent with the receptive female vs. the intact male

When the time spent in the incentive zones (intact male and sexually receptive female) at the 4 tests (pretest and 3 tests during treatment) in the 5 groups was evaluated with a three-factor mixed ANOVA there was no significant main effect of treatment ($F(4,41) = 1.30$, NS). There was an effect of test ($F(3,123) = 16.99$, $P < 0.001$), and of incentive ($F(1,41) = 5.57$, $P < 0.05$). There was no incentive x treatment interaction ($F(4,41) = 1.99$, NS), while there was a significant interaction test x incentive ($F(3,123) = 6.41$, $P < 0.001$). The three-way interaction test x incentive x treatment was not significant ($F(12,123) = 1.37$, NS). For readability, the illustration of the data is made in two figures, one for the time spent in the male incentive zone (Fig. 4) and another for the time spent in the receptive female incentive zone (Fig. 5).

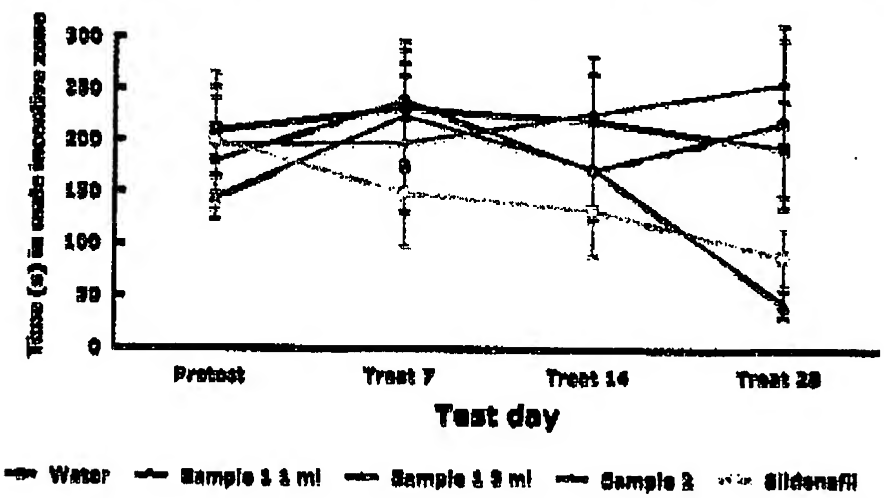

Figure 4. Mean ± S.E.M. time (sec) spent in the male incentive zone at the 4 tests in the 5 groups.

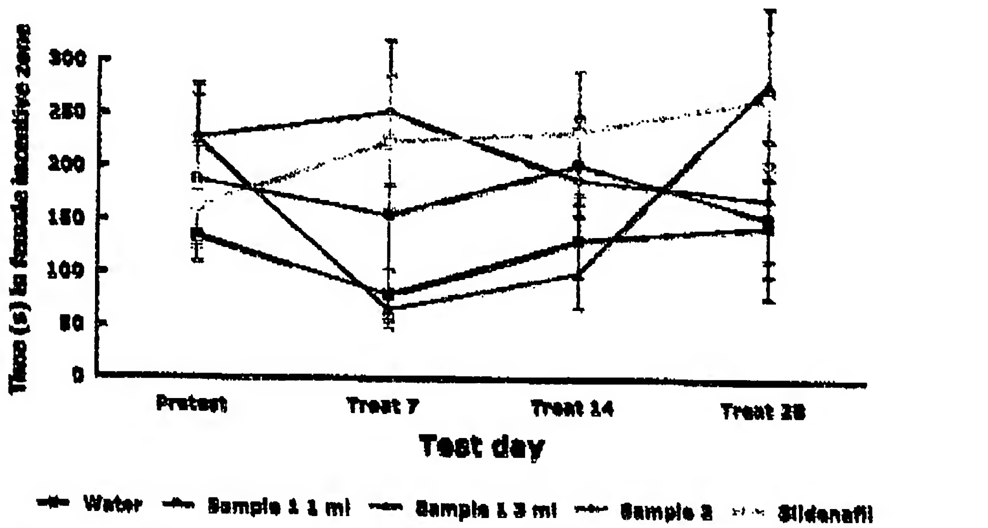

Figure 5. Mean ± S.E.M. time (sec) spent in the female incentive zone at the 4 tests in the 5 groups.

More detailed analyses of the data were performed for the test at treatment day 28. A mixed two-factor analysis of covariance was employed with the time spent in the male and female incentive zones at pretest as covariates. The within-groups factor was incentive (male – receptive female) and the between groups factor was treatment. There was no main effect of treatment ($F(4,39) = 0.29$, NS), and there was no significant difference between incentives ($F(1,39) = 3.00$, NS). To the contrary, the interaction treatment x incentive was significant ($F(4,39) = 3.50$, $P < 0.05$). Tests for simple main effect of incentive within each treatment showed that the experimental subjects spent more time in the receptive female incentive zone than in the male incentive zone only in the group treated with Sample 1, 3 ml ($F(1,40) = 8.10$, $P < 0.01$). In the other groups, there was no significant difference between the time spent in vicinity of the male incentive and that spent in the vicinity of the female incentive (all Ps > 0.09). As a curiosity it may be mentioned that the animals treated with sildenafil showed some tendency to spend more time in the receptive female incentive zone ($F(1,40) = 3.08$, $P = 0.087$). The results obtained in the analysis of covariance were substantiated by a comparison between the times spent in the receptive female and male incentive zones at each treatment with the Wilcoxon test. Again, there was a significant difference in the group treated with Sampe 1, 3 ml ($z = 2.31$, $P < 0.05$), but not in the other groups (Ps > 0.11). The time spent in the male incentive zone after the different treatments was then evaluated with test for simple main effect. There was a significant effect ($F(4,40) = 4.16$, $P < 0.01$). However, the Tukey HSD test revealed that no treatment differed from water. The significance was due to a difference between Sample 1, 3 ml, and Sample 2. The time spent in the female incentive zone after the different treatments was also evaluated with test for simple main effect. There was no significant effect ($F(4,40) = 1.46$, NS). Finally, we subjected the data to a nonparametric analysis with the Mann-Whitney U-test where all treatments were compared to water both with regard to the time spent in the male and in the female incentive zone. It now turned out that the group given Sample 1, 3 ml, spent less time in the male incentive zone than animals in the group given water ($U = 11$, $P < 0.01$). No other significant difference was obtained, although the sildenafil group was of borderline significance ($U = 22$, $P = 0.06$).

With regard to the time spent in the female incentive zone there was no significance (all $Ps >$ 0.12). Data are found in Fig. 6.

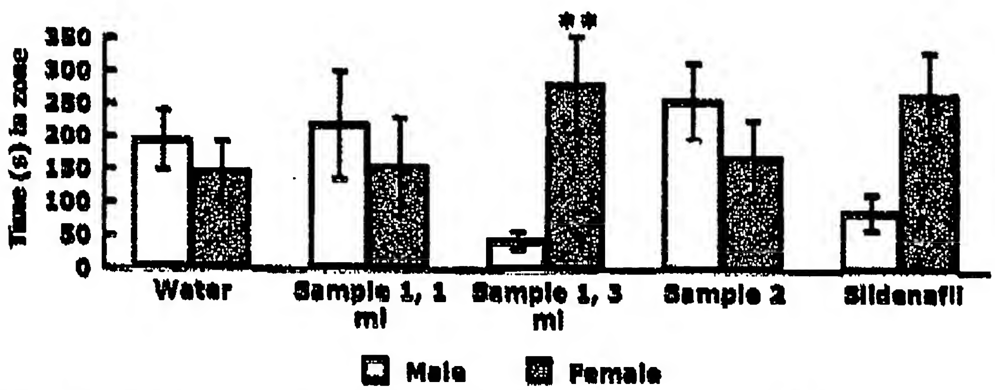

Figure 6. Time spent in the incentive zones at the test on treatment day 28. Data are mean ± S.E.M. **, different from the male, $P < 0.01$. Significance levels of differences between incentives within each treatment are based on tests for simple main effects.

1.1.3 Number of visits to the incentive animals

Three-factor mixed ANOVA of the number of visits to the incentive animals at the 3 test occasions showed a main effect of test ($F(3,123) = 11.74$; $P < 0.001$). There was no effect of incentive ($F(1,41) = 0.25$; NS) or of treatment ($F(4,41) = 0.46$, NS). The interactions test x treatment ($F(12,123) = 0.89$, NS), and incentive x treatment ($F(4,41) = 1.24$, NS) were nonsignificant. This was also the case for the interactions test x incentive and test x incentive x treatment ($F(3,123) = 0.41$, NS and $F(12,123) = 0.89$, NS, respectively). These results show that none of the treatments affected the number of visits to the incentives. In fact, the only effect obtained was that the number of visits was higher on the pretest than on the later tests. For readability, data are illustrated in two figures, one for the number of visits to the male incentive (Fig. 7) and one for visits to the female incentive (Fig. 8).

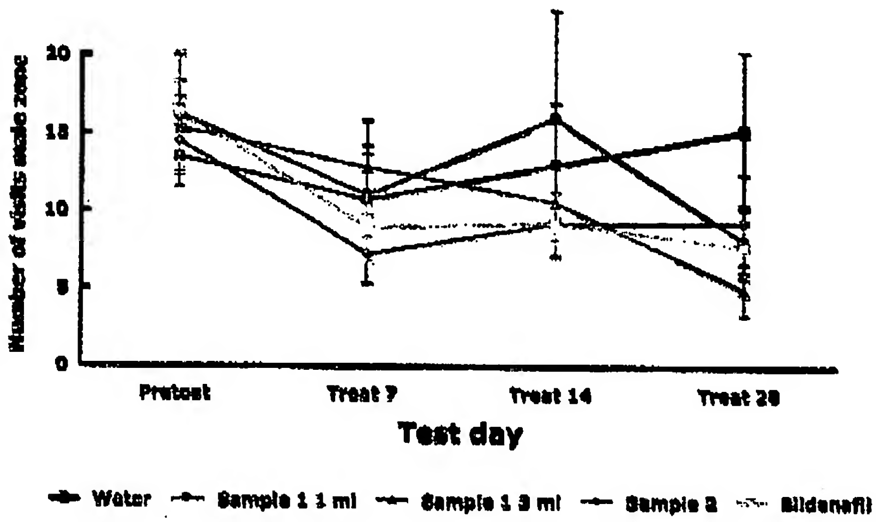

Figure 7. Mean ± S.E.M. number of visits to the male incentive.

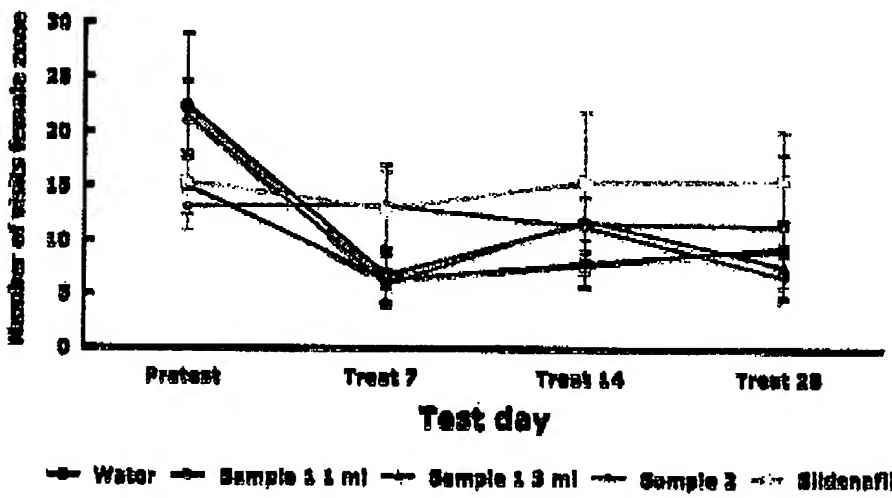

Figure 8. Mean ± S.E.M. number of visits to the female incentive.

The number of visits to the incentive animals was analysed in more detail for data from the test on treatment day 28 in a way similar to that used for the time spent in the

incentive zones. No effect of treatment ($F(6,76) = 1.54$, NS) or of incentive ($F(1,76) = 1.80$, NS) was found. Likewise, the interaction treatment x incentive was nonsignificant ($F(6,76) = 1.82$, NS). Data are shown in Fig. 9.

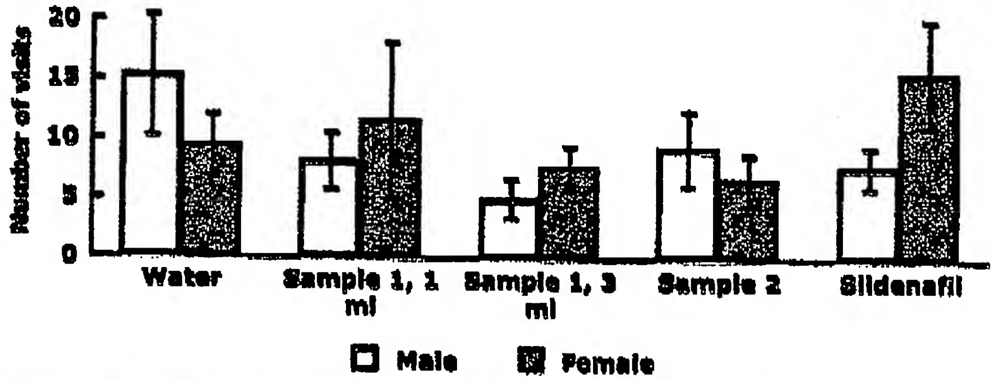

Figure 9. Mean ± S.E.M. of the number of visits to the incentives at the test on treatment day 28.

1.1.4 Ambulatory activity

With regard to the distance moved during the test, two-factor ANOVA with test as within groups factor and treatment as between groups factor did not detect any difference between treatments ($F(4,41) = 1.21$, NS). There was a difference between tests, though ($F(3,123) = 17.92$, $P < 0.001$), but no interaction test x treatment ($F(12,123) = 1.03$, NS). These data show that the treatments did not affect a sensitive indicator of general activity. Activity was somewhat higher at the first test than at the others, but this reduction in activity was independent of treatment. Data are found in Fig. 10.

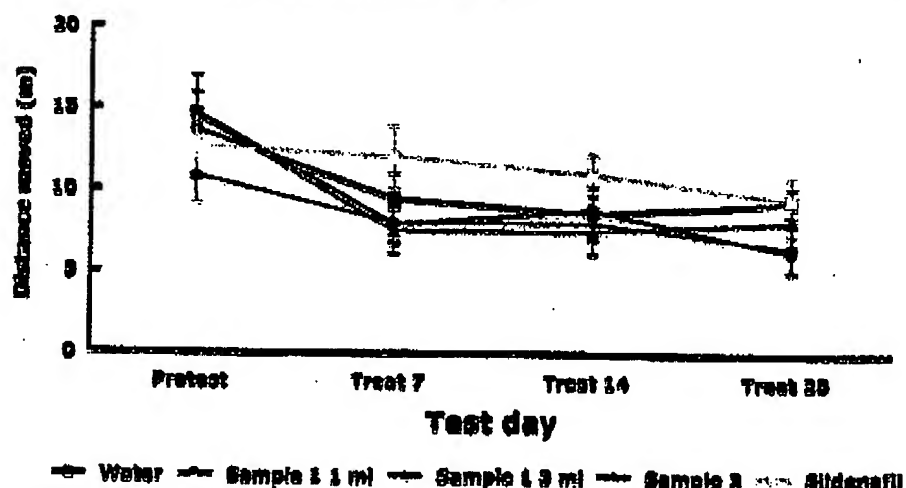

Figure 10. Ambulatory activity expressed as distance moved (in meters) during the sexual incentive motivation test in 5 groups of male rats at 4 tests.

Similar results were obtained when another indicator of motor function, the mean velocity of movement while moving, was analyzed. There was no effect of treatment, ($F(4,41) = 0.41$, NS) but there was an effect of test ($F(3,123) = 12.76$, $P < 0.001$). The interaction treatment x test turned out to be nonsignificant ($F(12,123) = 0.77$, NS). It is again concluded that there was no treatment effect on velocity of movement but there was a reduction between the pretest and the following tests. Data are illustrated in Fig. 11.

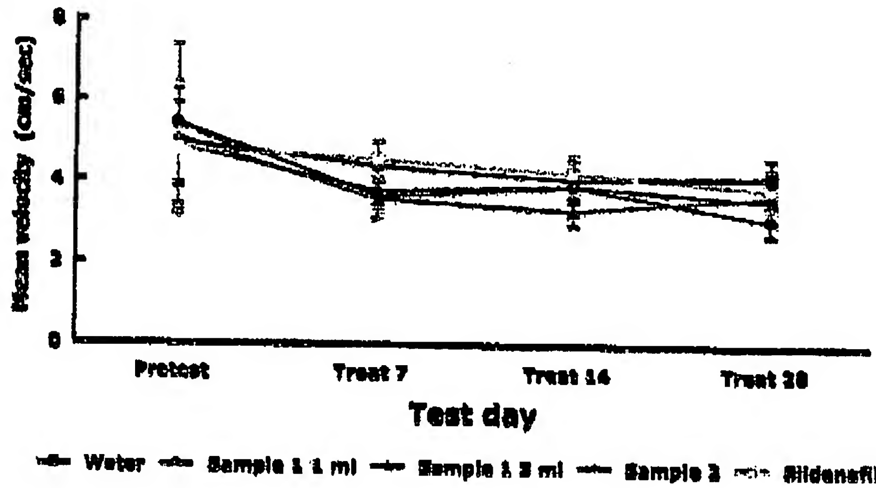

Figure 11. Ambulatory activity expressed as mean velocity of movement while moving (in cm/s) during the sexual incentive motivation test in 5 groups of male rats at 4 tests.

Finally, the time spent in inactivity (not moving) was evaluated. Again, there was no treatment difference ($F(4,41) = 0.48$, NS) while the tests differed ($F(3,123) = 9.60$, $P <$ 0.001). The interaction treatment x test was not significant ($F(12,123) = 0.85$, NS). Data are shown in Fig. 12. As was the case with the distance moved and the mean velocity of movement, these data show that activity was higher at the pretest than at the other tests while there was no effect of treatment. It seems safe to conclude that none of the treatments affected general activity.

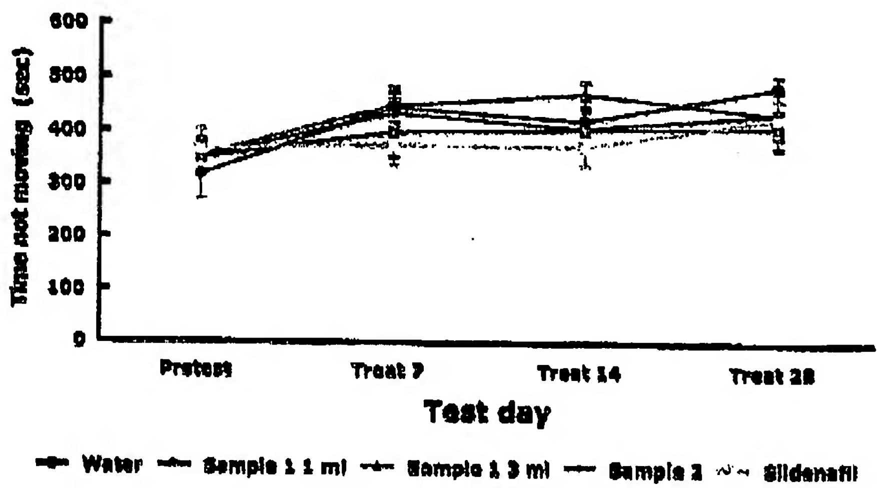

Figure 12. Ambulatory activity expressed as time not moving during the sexual incentive motivation test in 5 groups of male rats at 4 tests.

1.2 Copulatory behavior

Most parameters of sexual behavior can be obtained only from animals displaying the behavior. Since the old males employed in this study displayed very little sexual behavior the description of their behavior must be limited to measures that can be obtained regardless of absence or presence of copulatory activity. These are the proportion of animals displaying mounts, intromissions and ejaculation and the number of mounts and intromissions displayed.

Chi-square tests revealed that the groups did not differ with regard to the proportion of animals displaying at least one mount, intromission, or ejaculation at any test. There is no sign of any drug effect. Data are illustrated in figures 13 – 15.

Analysis of the number of mounts and intromissions revealed that the median was 0 for all groups at all tests. Since most of the animals had a value of 0 a normal distribution of the data is excluded, and it would consequently be inappropriate to employ the mean. Data concerning the number of mounts and intromissions are not illustrated.

The length of the erect penis was measured in the few subjects that displayed mounting and intromission. Three animals in the group treated with water displayed mounts, intromissions and ejaculation. Of these, 2 rats ejaculated on 2 tests, and the third ejaculated only at the pretest. One rat in the group treated with sildenafil ejaculated at the pretest but not at any of the treatment tests. No meaningful comparison between treatments can be made under these circumstances. As a matter of illustration, I have lumped all data together for a comparison of erection length at mount, intromission and ejaculation. For each rat at each test, the mean length was calculated for all mounts and all intromissions. The means of the individual means are illustrated in Table 1. As can be seen, the erect penis was longer

immediately after withdrawal from an intromission than it was during a mount. It was a tendency to be still longer after ejaculation. Statistical evaluation was not made since the same animals were included more than once.

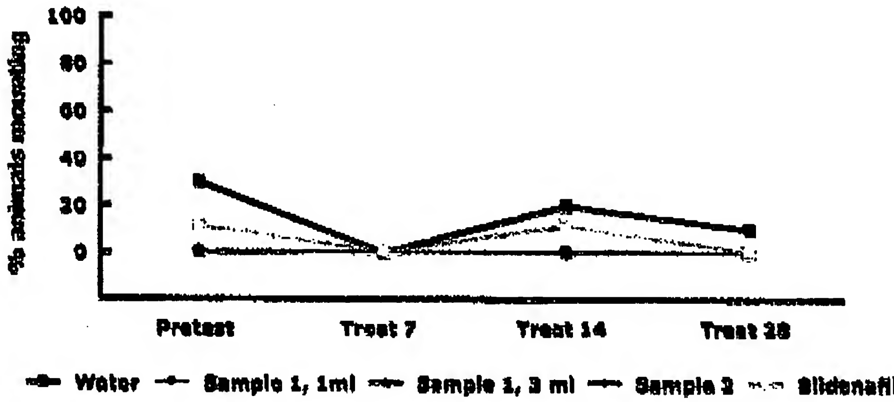

Figure 13. Proportion (expressed as percent) of animals displaying at least one mount at the 4 tests.

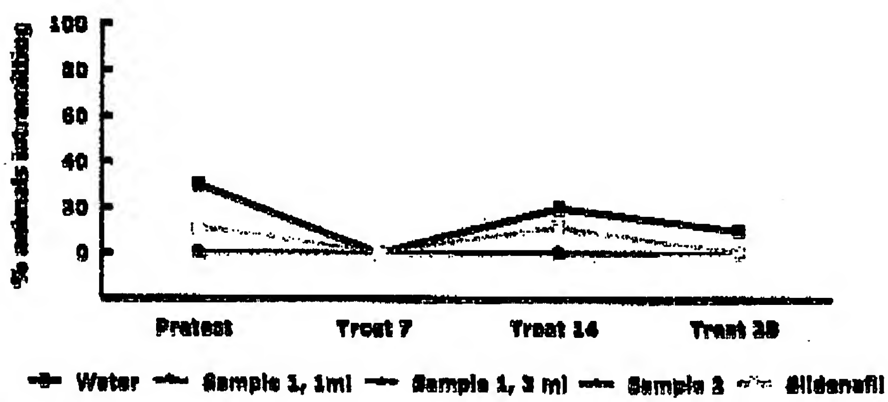

Figure 14. Proportion (expressed as percent) of animals displaying at least one intromission at the 4 tests.

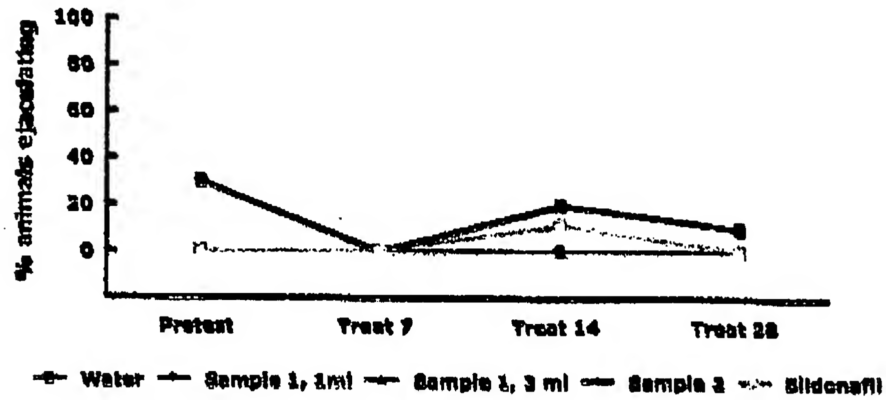

Figure 15. Proportion (expressed as percent) of animals displaying ejaculation at the 4 tests.

Table 1. Length of the erect penis during mounts and immediately after withdrawal from an intromission or ejaculation.

	Mean ± SEM
Mount	0.36 ± 0.04
Intromission	0.55 ± 0.03
Ejaculation	0.63 ± 0.12

Notes: Length is expressed in arbitrary units (mm on the projection screen).

Discussion

The data from the sexual motivation test show that none of the treatments affected any indicator of general activity, viz. distance moved, speed of movement or time spent without moving. To the contrary, the large dose of Sample 1, 3 ml, showed a clear tendency to enhance sexual motivation. When we analyzed the data obtained after 28 days of treatment, the preference score in this group was above that of animals treated with water. Furthermore, animals given Sample 1, 3 ml, spent more time in the vicinity of the receptive female than in vicinity of the male. Such a difference was not observed in any other group. However, the time spent in the female incentive zone was not significantly increased by this treatment. This means that the criteria for a clear-cut motivational effect are not satisfied. Thus, Sample 1, 3 ml, seems to enhance sexual motivation, but this suggestion must be regarded as preliminary. An important observation is that the possible motivational effect was not associated with

alterations in general activity. Although present data are only suggestive, the tendency to enhance sexual motivation is very interesting. Very few compounds have clear-cut motivational effects, so even a tendency to effect is quite remarkable. Indeed, of all the compounds we have tested, only some antagonists at the adrenergic α_2 receptor have a stimulatory effect on sexual motivation (Viitamaa et al., 2006).

Sildenafil showed a nonsignificant tendency to increase sexual motivation. This may be a spurious effect, since there are no clinical or experimental data suggesting that sildenafil enhances sexual motivation. There is limited evidence that it may facilitate some aspects of male rat copulatory behavior (Ferrari et al., 2002; Giuliani et al., 2002; Ottani et al., 2002), but that does not necessarily mean that motivation is affected.

The low dose of Sample 1 as well as Sample 2 failed to affect sexual motivation, and there was not even a tendency for an effect. This observation suggests that the activity in neural circuits involved in motivational processes were not modified by these treatments.

Copulatory behavior was almost absent in these old animals, despite extensive protesting with sexually receptive females. Pretests were performed twice and sometimes three times per week, giving these animals an intense exposure to females. At the last of these tests, copulatory activity was higher than at the experimental pretest, performed after one week of rest. The low activity observed at that test persisted throughout the experiment. This informal observation suggests that old males need frequent exposure to females if they are to display any copulatory behavior at all. An interesting observation is that these old males did not approach a sexually receptive female more than another male in the tests for sexual motivation. Thus, the males were not attracted to a sexual incentive (the female) any more than they were attracted to a social incentive (another male). An absence of sexual motivation can easily explain the absence of copulatory behavior. If the male is not attracted to the female, then there is no reason to believe that he would engage in copulatory behavior with her. This notion is further reinforced by data obtained from a group of young rats run simultaneously with the old ones. The young animals were not only attracted to a sexually receptive female, but they also copulated with her (data not shown). An interesting question that needs to be addressed, then, is why the animals given Sample 1, 3 ml, and which approached the receptive female at the test on day 28 of treatment, did not copulate. At present, only speculations can be made. The most reasonable of these is that while the treatment was efficient for enhancing sexual motivation it was not efficient enough for activating the copulatory reflexes in these old animals. Unfortunately, sexual motivation has not been studied in animals of similar age before, and data on copulatory behavior of rats having their first encounter with a female at an age of 18 months are extremely scarce (Clark, 1995). Due to this, it is not easy to integrate our observations with any existing literature. However, treatments that modify sexual motivation without altering copulatory behaviors have been observed in young, sexually active rats, showing that there is no necessary association between drug effects on sexual motivation and on copulatory behavior.

CONCLUSIONS

In sum, the findings of the present study show that Sample 1 (anti-endothelial NO synthase antibodies, ultra-low doses for oral administration), administered in the volume of 6.6 ml/kg for 4 weeks, stimulates sexual motivation in old, sexually inactive male rats without inducing any copulatory behavior. Perhaps, a longer treatment, or treatment with a still larger dose, could activate copulatory behavior in addition to enhancing sexual motivation.

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Sexual incentive motivation in old male rats: The effects of sildenafil and a compound (Impaza) stimulating endothelial NO synthase

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Abstract

Several proerectile drugs act on the nitric oxide–cyclic guanosine monophosphate pathway, which is known to influence on copulatory behavior. In the present study we evaluated the effects of two proerectile compounds, one (Impaza) acting on endothelial nitric oxide synthase and the other (sildenafil) on phosphodiesterase 5, on sexual incentive motivation in male rats displaying a spontaneously low level of motivation and copulatory behavior. About 20 months old male Fisher 344 rats were tested in a procedure for evaluating the intensity of sexual incentive motivation and in standard mating tests. For comparison, a group of young (about 4 months) Fisher 344 males was tested in parallel. This group did not receive any drug treatment. Impaza was administered in two doses daily for 28 days, and sildenafil was given at a dose of 3 mg/kg twice a week during 28 days. Tests for sexual incentive motivation and copulatory behavior were performed immediately before the beginning of drug treatments, and on days 7, 14 and 28 of treatment. All treatment groups displayed a very low level of copulatory behavior and a sexually receptive female was not a more powerful incentive than another male at the tests performed before and on days 7 and 14 of treatment. On day 28 of treatment, the group treated with Impaza, 3 ml, displayed a preference for the sexually receptive female, while no such preference was found in the other groups. Furthermore, the preference score was above that of controls in this group. Both Impaza, 3 ml, and sildenafil reduced approach to the male in the test for sexual incentive motivation, suggesting that social motivation was reduced. These data suggest that compounds affecting the nitric oxide–cyclic guanosine monophosphate pathway may modify both sexual and social motivation in old rats.

Keywords: Proerectile drugs; Sexual incentive motivation; Copulatory behavior; Aging rats; NO; PDE5; sildenafil

1. Introduction

Sexual behavior is not possible at a distance. At least two individuals need to be in close proximity before any sexual interaction can take place. Thus, before sexual behaviors can be executed it is necessary to localize and approach a potential mate. The intensity of approach behaviors is generally believed to be determined by the intensity of sexual motivation (see Ågmo, 1999, 2003; Ågmo et al., 2004; Hetta and Meyerson, 1978; Meyerson and Lindström, 1973 for discussions of this issue). Research on the neural control of sexual motivation has become increasingly important because of the large amount of clinical data showing that low sexual desire is a human problem with an unexpectedly high prevalence (see e.g. [illegible] et al., 1995; Laumann et al., 1999; [illegible], 1998). Consequently, the need for an efficient pharmacological treatment is widely recognized, but there is at present no treatment with established effects and there is no drug approved for the pharmacotherapy of hypoactive sexual desire disorder.

In men, erectile dysfunction may be associated with low sexual desire (Corona et al., 2004; Lewis et al., 2004), and it has been reported that treatment of the erectile deficiency may restore sexual desire ([illegible] et al., 1999), suggesting that proerectile drugs indirectly may enhance sexual motivation. Recent data even suggest that some proerectile drugs may have a direct effect on motivational mechanisms. For example, it has been proposed that nitric oxide plays a role in the mechanisms involved in sexual motivation in addition to its well established importance for erection. An early study showed that the nitric

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oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (NAME) reduced intromissions and ejaculation in male rats but enhanced mounting (Hull et al., 1994). This suggests that erection was adversely affected. In a test for sexual motivation, no effect was found. Different results have, however, been reported in more recent studies. When the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (NMMA) was administered to the medial preoptic area by reverse dialysis a reduced number of mounts was found. Interestingly, the intromission ratio (the proportion of mounts ending in vaginal penetration, intromission) remained unaffected (Sato et al., 1998). The intromission ratio is sensitive to changes in erectile function, and these data suggest that centrally reduced nitric oxide availability does not affect erectile capacity. Further evidence for a role of central nervous nitric oxide in male sexual behavior comes from a study in which NAME was infused into the medial preoptic area. Mounting was almost abolished in sexually inexperienced animals and severely reduced in animals with sexual experience (Lagoda et al., 2004). The results of these latter studies coincide in suggesting that nitric oxide is important not only for copulatory behavior but also for sexual motivation. The fact that many males did not copulate at all after treatment with NAME certainly suggests that sexual motivation was reduced or absent.

Many cellular actions of nitric oxide are dependent on the activation of guanylylcyclase and the subsequent formation of cyclic guanosinemonophosphate (cGMP). Nitric oxide responsive guanylylcyclase is widely distributed in the brain, including areas important for male sexual behavior (De Vente et al., 1998). It is, indeed, most likely that the effects on sexual behavior observed in the studies mentioned above are mediated by cGMP. Recent experimental data have confirmed this hypothesis (Sato and Hull, 2006).

Nitric oxide is also synthesized outside neurons, mainly through the action of endothelial nitric oxide synthase (eNOS). eNOS is present in blood vessels, including capillaries, and it has been shown that nitric oxide derived from eNOS diffuses into and affect adjacent neurons in a cGMP dependent way (Garthwaite et al., 2006). This makes it possible to envisage behavioral consequences of manipulations of eNOS. In fact, limited evidence for the importance of eNOS in sexual behavior comes from studies of mice lacking the gene for eNOS. Such mice ejaculate after fewer mounts and intromissions (Kriegsfeld et al., 1999). This suggests that the ejaculatory reflex was facilitated. No independent test for sexual motivation was performed.

cGMP is catabolized by a series of phosphodiesterases. One of these is phosphodiesterase 5. This enzyme is the target of several proerectile drugs, like sildenafil, tadalafil or vardenafil. The stimulatory effect of these drugs on erection is supposed to be localized to the corpora cavernosa (Uckert et al., 2006), but PDE5 has been found in several areas of the brain. Among the structures where the presence of PDE5 in large amounts has been established are the cerebellar Purkinje cells (Bender and Beavo, 2004). Several other brain areas, for example the olfactory bulbs and hippocampus express PDE5 to a much lesser degree (Lin et al., 2006). It is not impossible that this enzyme is also present and functionally relevant in brain areas related to sexual behavior. Concordant with this proposal is the fact that sildenafil affects several parameters of sex behavior when administered to male rats. In a group of males selected for unusually low intromission ratio and long intervals between intromissions the number of preejaculatory mounts, the ejaculation latency, and the postejaculatory interval were reduced while the intromission ratio was enhanced by sildenafil. In unselected rats, the only drug effect found was reduced ejaculation latency (Ottani et al., 2002, Ottani et al., 2002). Sildenafil has also been shown to enhance inter-male mounting in sexually experienced but not in inexperienced rats (Ferrari et al., 2002). This last observation suggests that sildenafil has some stimulatory effect on sexual motivation. A study in rams also indicates that sildenafil may enhance the motivation to engage in copulatory behavior (Cayan and Kaya, 2005). In this study sildenafil, at a dose of 100 mg per animal, was administered intrarectally to rams with low or high sexual activity according to performance on a screening test. Sildenafil enhanced the number of ejaculations in both groups. This effect was interpreted as suggestive of increased sexual motivation.

The data presented in the preceding paragraphs suggest that eNOS or nNOS (neural nitric oxide synthase) activity and cGMP may influence male sexual behavior. Consequently, compounds modifying the activity of eNOS or nNOS as well as phosphodiesterase inhibitors should be effective. Considerable evidence exists for a role of nNOS (see above), but data with regard to the role of eNOS in male sexual behavior are far from abundant. Likewise, the effects of phosphodiesterase inhibition, particularly inhibition of PDE5, have only been the subject of a handful of studies. More data with regard to the potential role of eNOS and PDE5 inhibition are needed before any firm conclusion as to their role in male sexual behavior can be drawn. The purpose of the present experiment is to contribute to that end. We evaluated the motivational effects of a compound, Impaza, acting on eNOS. Impaza is an antibody to the C-terminal fragment of eNOS, but paradoxically it has been reported to enhance the activity of endogenous eNOS when administered at extremely low doses. The compound is effective as monotherapy for erectile deficiency in the human and it also increases the efficacy of PDE5 inhibitors on combined treatment (Martyushev-Poklad et al., 2005; Mazo et al., 2004). Its effects on sexual motivation and copulatory behavior are entirely unknown. In order to determine possible motivational effects of PDE5 inhibition, one group of rats was treated with sildenafil. Both compounds were evaluated in a procedure especially designed for the evaluation of sexual incentive motivation (Ågmo, 2003; Ågmo et al., 2004). In addition, effects on copulatory behavior were determined in standard mating tests.

It is generally believed that male rats with low sexual activity are more sensitive to stimulatory actions of drugs than rats with high sexual activity. This belief was confirmed in the study of sildenafil mentioned above (Ottani et al., 2002). Thus, in order to maximize the possibility of detecting potential effects of the treatments employed in the present experiment we used rats with low sexual activity. Rather than selecting a subsample of rats with such activity from a larger pool, we decided to use rats whose "normal" sexual activity is low. It is known that rats older than 20 months display much reduced sexual activity (Clark,

1995; Roselli et al., 1993; Smith et al., 1992; Isai et al., 1998). Thus, we used rats around 20 months of age.

2. Materials and methods

2.1. Subjects

Male Fisher 344 rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN) through the Aged Rodent Colonies of the National Institute on Aging. The experimental males were 18 months old when arriving to the laboratory, and about 20 months old when drug treatments were initiated. Twelve additional Fisher 344 rats from the same provider were about 3 months old when arriving to the laboratory and about 4 months of age when behavioral observations were begun. The young animals were included for comparison only, and they did not receive any drug treatment.

All subjects were housed in pairs in Macrolon cages under a reversed light/dark cycle (12:12 h, lights on 2300) in a room with controlled temperature (21 ± 1 °C) and relative humidity (55 ± 10%). Rodent pellets (RM1(E), Special Diet Services, Witham, Essex, UK) and tap water were freely available.

Male (300 g upon arrival) and female (250 g upon arrival) Wistar rats (Scanbur, Sollentuna, Sweden) were used as incentive animals in the test for sexual motivation. Similar females were used as copulation partners. These males and females were housed in the same room and under the same conditions as the experimental males.

All females were ovariectomized under isoflurane anesthesia at least 2 weeks before use. Before all testing sessions, estrus was induced by administration of estradiol benzoate, 25 μg/rat, followed by progesterone, 1 mg/rat, 48 h later. Females were used between 4 and 8 h after the progesterone injection. Both steroids were purchased from Sigma (St. Louis, MO, USA). They were dissolved in peanut oil and injected subcutaneously in a volume of 0.2 ml/rat.

The experimental procedures employed were approved by the Norwegian Committee for Ethics in Research on Animals and were in agreement with the European Union council directive 86/609/EEC.

2.2. Apparatus

Sexual incentive motivation was evaluated in a rectangular arena (100 × 50 × 45 cm high) with rounded corners. At the long sides were two diagonally opposed openings (25 × 25 cm). On the outside of each of these openings a small (15 × 25 × 25 cm high) box containing an incentive rat could be fitted. The animal inside the cage was separated from the arena by a double wire net. The mesh size was 12 × 12 mm and the two nets were separated by 10 mm. This meant that the animals could hear, see and smell each other while no direct physical contact was possible. Video cameras, fixed to the ceiling above the center of each arena, were connected to a computer and a videotracking system (Ethovision Pro, Noldus, Wageningen, the Netherlands) determined the experimental subjects' position with a frequency of 5 Hz. A virtual zone of 21 × 38 cm was defined outside each of the openings in the arena wall, and the time spent in these zones and the number of entries into them were determined. In addition, the distance moved during the test, the mean velocity of movement while moving and the time not moving were calculated. A more detailed description of the testing environment can be found in Ågmo (2003) and Ågmo et al. (2004).

Copulatory behavior was observed in rectangular sheet steel cages (40 × 60 × 40 cm high) with a Plexiglas front and glass floor. Under the floor there was a mirror tilted in an angle of 45°. This allowed for a ventral view of the experimental subjects. All test sessions were videotaped for later analyses.

The test for sexual incentive motivation was performed in a room lit by dim, white light. Light intensity at the level of the arena floor was about 5 lx. Tests for copulatory behavior were performed in an adjacent room. Here, the light intensity was around 25 lx, as measured at floor level in the mating cage.

2.3. Drugs

Sildenafil citrate was obtained as commercial tablets (Viagra®, Pfizer, USA) containing 25 mg. The tablets were crushed in a mortar and then dissolved in physiological saline. The reason for using commercial tablets rather than the pure compound was that we wanted to make the drug treatment as similar to the clinical use of sildenafil as possible. Antibodies to C-terminal fragment of endothelial NO synthase (mixture of homeopathic dilutions C12, C30, C200, Impaza®, OOO NPF Materia Medica Holding, Moscow, Russia) was provided as a ready-to-use solution in distilled water. The actual concentration of antibodies is not known, but the solution used here is identical to the one employed in clinical practice.

2.4. Design and procedure

The males were familiarized to the motivation test arenas during 3 sessions of 10 min each separated by 24 h. One of the incentive animal boxes contained a sexually receptive female and the other an intact male. The incentive animals were drawn from a lot of 10–12 rats maintained for the purpose, and were sexually experienced. This means that each incentive animal was used more than once. A few days after familiarization to the motivation test, screening tests for copulatory behavior were initiated. Up to this point, all animals were sexually naive. At each test, the male was placed in the testing cage 5 min before the introduction of a sexually receptive female. The latency to the first mount with pelvic thrusting and the latency to the first vaginal penetration (intromission) as well as the number of mounts and intromissions before the first ejaculation were recorded. The ejaculation latency (time from the first intromission until ejaculation) and the postejaculatory interval (time between ejaculation and the next intromission) were also determined. The test was ended at the end of the postejaculatory interval, or 15 min after the introduction of the female if no intromission occurred, or 30 min after the first intromission if ejaculation had not occurred, or 15 min after ejaculation if no postejaculatory intromission was performed before that time. These tests were repeated twice weekly for 3 weeks. The same

criteria for ending the test were employed also at the tests performed during drug treatment. The likelihood of initiation of copulatory behavior after the first 10 min exposure to a female is very low (see e.g. Ågmo, 1999), so a longer test would probably not increase the proportion of sexually active males.

At the end of the training period, the subjects were randomly assigned to one of four groups of 10 rats each. One group was treated with distilled water, 2 ml/kg and day for 28 days. The second and third groups were given Impaza, 1 and 3 ml/kg and day respectively, for 28 days. The fourth group was treated with sildenafil, 3 mg/kg in a volume of 2 ml/kg, twice weekly for 28 days. On the other days, these animals received distilled water, 2 ml/kg. All treatments were given orally by gavage. Care was taken to avoid leakage from the mouth. Again, oral treatment was used in order to be as close as possible to the clinical use of the compounds. This was also the rationale for giving sildenafil twice weekly rather than daily. Most men using sildenafil belong to an age group where the frequency of intercourse rarely exceeds twice a week. The sildenafil dose is within the range of doses that previously has been found to be effective in studies of copulatory behavior in young rats (Ferrari et al., 2002).

The experimental phase started with a 10 min test for sexual motivation immediately followed by a test for copulatory behavior. The following morning, drug administration was started. Tests were then performed at days 7, 14 and 28 of drug treatment. In parallel to the 4 experimental groups, a group of 12 young rats was tested for sexual incentive motivation and copulatory behavior in exactly the same way as the experimental rats. The young animals had been subjected to familiarization to the motivation arena and screening for copulatory behavior together with the experimental rats.

2.5. Statistics

Sexual motivation was quantified in several ways. Most important for evaluating the sexual incentive value of the receptive female is the preference score (time spent in the female incentive zone/(time spent in the female incentive zone + time spent in the male incentive zone)). The preference score at the pretest was analyzed with ANOVA with treatment as factor. The score obtained at day 28 of treatment was evaluated with an analysis of covariance with the preference score at pretest as covariate. With regard to the time spent in the female and male incentive zones as well as the number of visits to them, the pretest data were evaluated by two-factor ANOVAs with repeated measures on one factor, the within group factors being incentive (male, female) and the between-groups factor being treatment. Data from each of the tests performed during the treatment period were evaluated with an analysis of covariance with treatment as between-groups factor, incentive as within-groups factor and pretest time spent in the male and female zones as covariates. In case of significant interaction, tests for simple main effects of incentive within each treatment as well as effects of treatment within each incentive were performed (Winer et al., 1991). Indices of ambulatory activity were analyzed as the preference score.

Because of the low sexual activity displayed during the experiment, the analyses of the data from the copulatory behavior tests had to be limited to the proportion of subjects displaying mount, intromission or ejaculation. Treatments were compared with the chi-square test.

Data from the young group was not included in any of the abovementioned analyses. However, data obtained at the session immediately before the initiation of drug treatment to the experimental rats (pretest) were used for comparing the young group with the older rats. The t-test was employed for the analysis of the preference score and for all indices of ambulatory activity. The time spent in the incentive zones as well as the number of visits to them were evaluated with a mixed two-factor analysis of variance. The within-groups factor was incentive (male, female) and the between-groups factor was age (young, old).

Pretest data were also used for comparing copulatory behavior in young and old rats. The proportion of animals displaying mount, intromission and ejaculation was analyzed with the Fisher exact probability test. The number of mounts and intromissions was evaluated with Mann–Whitney's U-test. The reason for employing a non-parametric test was that most animals had a value of 0 on both behavior patterns, making the distribution of the data dramatically skewed. The latencies and the postejaculatory interval were obtained from so few animals that a meaningful analysis was impossible.

3. Results

One animal in the group to be treated with Impaza, 1 ml, died before the beginning of drug administration. The same occurred to a rat in the group to be given Impaza, 3 ml. The local veterinarian performed autopsy but failed to identify the causes of death. It was attributed to old age. Two other animals lost weight and showed signs of bad health before the last drug treatment test. One belonged to the group given Impaza, 1 ml and another to the group given sildenafil. These animals were eliminated from all analyses.

3.1. Sexual motivation

3.1.1. Pretest

There were no significant group differences in preference score at the pretest ($F_{3,32}$ = 0.75, NS). Likewise, there was no group difference in time spent in the incentive zones ($F_{[illegible]}$ = 0.56, NS), no difference between incentives ($F_{[illegible]}$ = 0.23, NS) and no interaction incentive × treatment ($F_{[illegible]}$ = [illegible], NS). Furthermore, none of the groups spent more time in the receptive female incentive zone than in the male incentive zone or had a preference score significantly above chance level. Data are illustrated in Fig. 1 A and B.

The number of visits to the incentive zones did not differ between groups ($F_{3,32}$ = 0.92, NS) or between incentives ($F_{[illegible]}$ = 1.43, NS). The interaction between group and incentive was also nonsignificant ($F_{3,32}$ = 0.44, NS). These data are not shown. The results from the pretest establish that the experimental groups did not differ significantly before the beginning of drug treatment.

When the pretest data from young and old rats were compared, it was found that the preference score was significantly

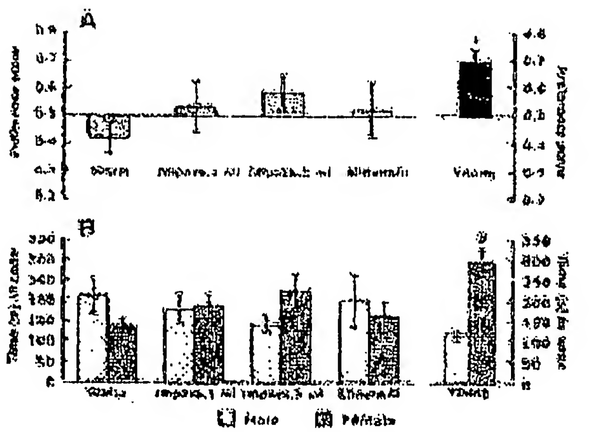

Fig. 1. A. Preference score in old male rats (left panel) obtained at the test performed before the beginning of drug treatments, the pretest. The right panel shows data from a group of 4 months old rats tested in parallel with the experimental groups. B. Time spent in the male and female incentive zones in the pretest in the experimental groups (left panel) and in a group of young rats (right panel). Data are mean+SEM. *, different from no preference, i.e. a score of 0.5, P<0.05. §, different from the male incentive, P<0.05. For further details, see text.

larger in young than in old rats (t_{35}=2.64, P<0.05). Furthermore, the score in the young rats was significantly above chance (t_{11}=5.43, P<0.001), showing that these animals preferred the female over the male (see Fig. 1A, right panel). Concerning the time spent in the incentive zones it was found that there was no main effect of age ($F_{1,46}$=2.35, NS). The main effect of incentive ($F_{1,46}$=7.10, P<0.01) as well as the interaction age × incentive ($F_{1,46}$=8.50, P<0.01) were significant. Tests for simple main effects of group within each incentive revealed that there was no difference between young and old rats with regard to the time spent in the male incentive zone ($F_{1,46}$=2.83, NS) while there was a difference in the time spent in the female incentive zone ($F_{1,46}$=12.72, P<0.001). Tests for simple main effects of incentive within groups showed that the young rats spent more time in the female than in the male incentive zone ($F_{1,46}$=10.45, P<0.01). There was no difference between incentives in the old rats ($F_{1,46}$=0.06, NS). Data are illustrated in the right panel of Fig. 1B. Analysis of the number of visits to the incentives showed that the young rats made more visits than the old rats ($F_{1,46}$=7.66, P<0.01). There was no difference between the number of visits made to the male and female incentives ($F_{1,46}$=2.60, NS) and no interaction age × incentive ($F_{1,46}$=0.08, NS). Thus, the young rats were generally more active than the old rats.

The comparisons between young and old rats show that age did not influence approach to a social incentive (the males). In contrast, approach to a sexual incentive was much reduced in the old rats. Thus, sexual incentive motivation appears to be low or absent in such rats.

3.1.2. Tests during drug treatment

Since the young rats did not receive any drug treatment, no further mention is made of them. Consequently, the subsequent part of the results refers exclusively to data from the 20 months old rats.

The results from the tests at day 7 and 14 of treatment were similar to those obtained at the pretest. There was no significant difference between treatments, and the subjects did not show any preference for the sexually receptive female (all Ps>0.1?, data not shown). On test day 28, there was a significant difference between treatments with regard to the preference score ($F_{3,41}$=3.93, P<0.05). When treatment groups were compared to control, it turned out that the groups treated with Impaza, 3 ml, and sildenafil had a preference score above that of the control group (P<0.05). When the preference score obtained at each treatment was compared to no preference (a score of 0.5), it was found that only the group given Impaza, 3 ml, had a significant preference for the female incentive. Results are illustrated in Fig. 2A.

Analysis of the time spent in the incentive zones did not reveal any main effect of treatment ($F_{3,41}$=0.15, NS), or of incentive ($F_{1,41}$=2.67, NS) while the interaction treatment × incentive turned out to be significant ($F_{3,41}$=4.06, P<0.05). Tests for simple main effect of incentive within treatment showed that the subjects treated with Impaza, 3 ml, spent more time in the female incentive zone than in the male incentive zone ($F_{1,41}$=10.53, P<0.01). There was no significant difference between incentives within other treatments (Ps>0.08). When treatment effects within each incentive were analyzed, it turned out that the treatments differed with regard to the time spent in the male incentive zone ($F_{3,41}$=4.14, P<0.05) but not with regard to that spent in the female incentive zone ($F_{3,41}$=1.23, NS). *Post hoc* analyses revealed that the groups treated with Impaza, 3 ml, and sildenafil spent less time in the male incentive zone than

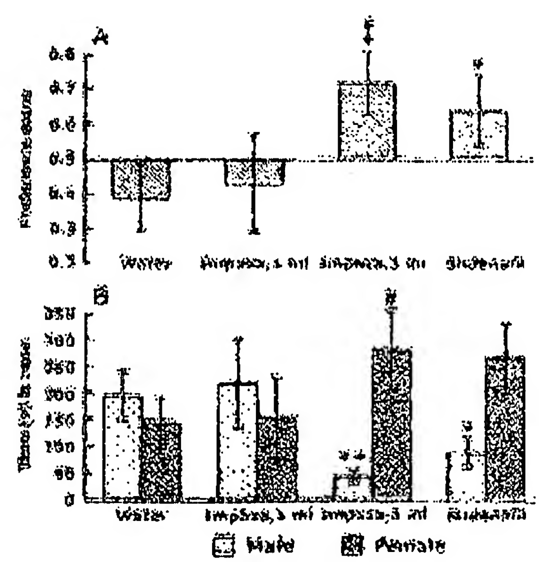

Fig. 2. A. Preference score in old male rats obtained at the test on day 28 of drug treatment. B. Time spent in the male and female incentive zones at day 28 of treatment. Data are mean+SEM. *, different from water, P<0.05. **, P<0.01. [illegible] different from no preference (a score of 0.5), P<0.05. §, different from male, same treatment, P<0.05.

controls, while there was no effect of the other treatments (Fig. 3B).

3.2. Ambulatory activity

Indices of general activity revealed that the groups moved a similar distance during the pretest ($F_{3,43}$=0.24, NS), had a similar velocity of movement while moving ($F_{3,42}$=0.37, NS) and spent about the same time not moving ($F_{3,42}$=0.33, NS) at that test. This was also the case at the test performed on day 28 of treatment (distance, ($F_{3,42}$=1.42, NS; velocity ($F_{3,42}$=0.86, NS), inactivity time ($F_{3,40}$=0.62, NS)). Thus, no treatment affected any of the parameters indicative of general activity. Data from the test performed on day 28 of treatment are shown in Fig. 3.

While the drug treatments failed to affect ambulatory activity, data from the pretest show that age had a profound effect. Comparison between the young and old animals with regard to the distance moved during the test shows that the young moved longer than the old animals (t_{46}=6.63, P<0.001). Likewise, the young animals moved faster as revealed by analysis of the mean velocity of movement while moving (t_{46}=3.15, P<0.01). In contrast, there was no difference between young and old rats with regard to the time spent without moving (t_{45}=1.27, NS).

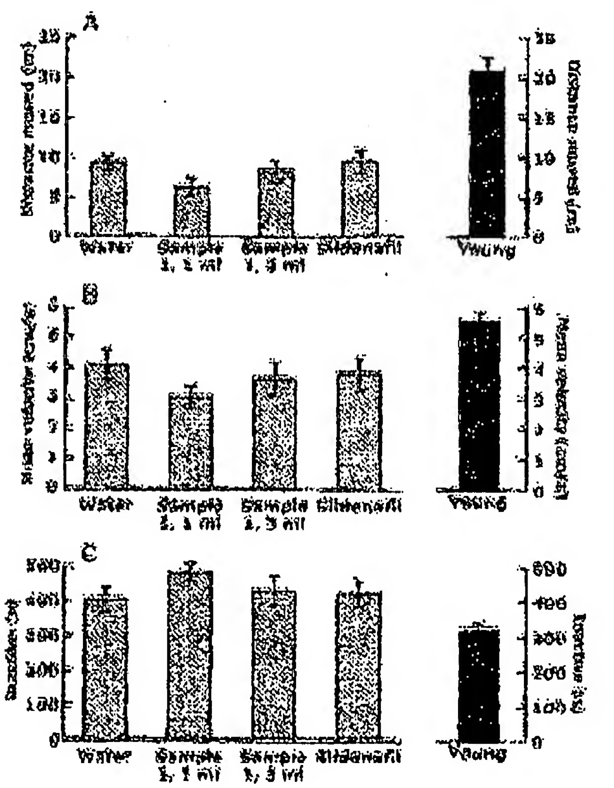

Fig. 3. Distance moved during the test performed at day 28 of treatment (A), mean velocity of movement (B) and time spent in inactivity (C) at that test. For comparison, data from a group of young rats tested in parallel with the experimental groups are shown to the right in each panel. Data are mean±SEM.

Table 1
Sexual behavior in male Fisher 344 rats about 4 months (Young) and about 26 months (Old) of age

Behavior	Young	Old
Proportion of animals displaying mount	58	8**
Proportion of animals displaying intromission	33	8
Proportion of animals displaying ejaculation	25	8
Number of mounts	8.1±3.3	1.2±0.78***
Number of intromissions	1.8±0.9	0.2±0.2*
Mount latency	345±70	80±52
Intromission latency	113±62	245±167
Ejaculation latency	487±85	453±105
Postejaculatory interval	430±32	362±86

Data are means±SEM. Latencies are expressed in s. **, different from young, P<0.01; ***, P<0.001.

These data show that the younger rats moved a longer distance because they moved faster than the old rats but not because they spent more time moving. Data are illustrated in Fig. 3, right panels.

3.3. Copulatory behavior

All experimental groups displayed a very low level of sexual behavior at the pretest. In fact, the number of rats displaying mount, intromission and ejaculation was so low that no meaningful analysis of these parameters could be performed. Despite the fact that the rats were randomly assigned to the treatment groups immediately before the pretest, it appeared that the group to be treated with water included more sexually active animals than the other groups. During the entire experiment, 3 animals in this group mounted, intromitted and ejaculated on at least one occasion. In the group given sildenafil, one rat achieved ejaculation at the test on day 14 of treatment. Otherwise no copulatory activity was observed in any group. At the test performed on day 28 of treatment, for example, one rat in the group treated with water mounted, intromitted and ejaculated. In the other groups, not one single animal mounted. Not surprisingly, the χ^2 test revealed that there was no significant group difference (all Ps>0.34).

When the pretest data from the young rats were compared to those from the old rats several differences were found. The proportion of young rats displaying at least one mount was significantly larger than that of the old rats (P=0.001, the Fisher test), and the number of mounts performed was superior in the young rats (Mann-Whitney's U=113, P<0.001). There was a borderline effect of age on the proportion of rats displaying intromission (P=0.051, the Fisher test) while the number of intromissions was larger in the young than in the old rats (Mann-Whitney's U=166.5, P<0.05). To the contrary, there was no significant difference with regard to the proportion of animals ejaculating (P=0.19, the Fisher test). This is due to the rather low number of young rats achieving ejaculation at this particular test. The low number of old rats displaying copulatory behavior made statistical comparisons of mount, intromission and ejaculation latencies as well as of the postejaculatory interval meaningless. Nevertheless, it can be maintained that about 4 months old Fisher 344 rats display a more intense sexual

behavior than rats 20 months old do. Data are summarized in Table 1.

4. Discussion

The Fisher 344 rats displayed very little sexual behavior when their first encounter with a sexually receptive female occurred at the age of 20 months. In fact, their sexual behavior was much inferior to that previously reported for Fisher rats having acquired extensive sexual experience when young. When tested at about the same age (21–22 months) between 30 and 40% of these rats displayed ejaculation (Chambers et al., 1991; Roselli et al., 1993). Substantial sexual activity has also been found in old, sexually experienced rats of other strains (Smith et al., 1992). The proportion of rats displaying ejaculation at pretest in the present study, 6%, was much lower than in the studies mentioned above. This observation suggests that acquisition of sexual experience when young enhances the likelihood of displaying sexual behavior when old. Indeed, the only other study in which sexually inexperienced animals were employed reported that only 16% ejaculated when tested at 18–19 months of age (Tsai et al., 1994). This is not much different from the results obtained with our 20 months old animals, supporting the notion that prior experience is a crucial determinant of sexual behavior in old rats. Independently of this, it can be concluded that our aim of testing Impaza and sildenafil in rats with spontaneously low sexual activity was fulfilled.

Not only did most of the old rats fail to display sexual behavior, but they also failed to show any signs of sexual incentive motivation. They did not spend more time in the vicinity of a receptive female than in the vicinity of another male. This is in sharp contrast to a wealth of data from young male rats, in which a preference for the receptive female has been solidly established in a variety of procedures (see Ågmo et al., 2004; Pfaff and Ågmo, 2002, for a discussion). It is probably so that the sexually receptive female's lack of incentive properties for old males is the cause of the absence of copulatory behavior in those males. As pointed out in the Introduction, sexual behavior is not possible at a distance, and if the female is unable to activate approach behaviors there is no way she can activate copulatory behaviors. Sexual incentive motivation was not evaluated in any of the studies with old rats mentioned above, but it is likely that the reduced proportion of males displaying copulatory behavior as well as the reduced intensity of that behavior in the few males who did display it are a consequence of reduced sexual motivation. The data from the group of young rats tested in parallel to the experimental subjects substantiate this notion. The young animals did not only show a more intense sexual incentive motivation than the old animals but also a more intense copulatory behavior. It seems, then, that old male rats constitute a good model for studying potential treatments for reduced or absent sexual incentive motivation.

Impaza, 3 ml, seemed to enhance sexual incentive motivation at the test performed on day 28 of treatment in the way that the experimental males approached the incentive female more than the incentive male. However, this was mainly due to a reduced intensity of approach to the male incentive. The time spent in the female incentive zone was increased compared to control, but not sufficiently for statistical significance, making it questionable whether the female's incentive value was enhanced by the drug treatment or not. Indeed, the continued absence of copulatory behavior shows that the incentive motivational properties of the female were not sufficient for making the males engage in copulatory activity. Nevertheless, it is possible that a longer treatment period would have succeeded in having more robust effect on incentive motivation and eventually also on copulatory behavior. Since the time course of the effects of Impaza on eNOS is not well known, this is pure speculation, however. In this context it is worthwhile to remember that much evidence shows that approach to a potential mate is controlled by mechanisms partly different from those controlling the execution of copulatory behavior (see e.g. Ågmo, 2002), and that male rats may display approach behavior even though copulation does not follow (Stone et al., 1935). To the contrary, copulation without preceding approach is impossible, as already pointed out.

Sildenafil did not significantly modify sexual incentive motivation although its effect on the preference score was of borderline significance ($P=0.09$). Like Impaza, 3 ml, it reduced the time spent in the male incentive zone at the test performed on day 28 of treatment without producing any significant increase in the time spent in the female incentive zone. This observation suggests that the effect seen with Impaza, 3 ml, is not spurious but somehow related to enhanced activity of NO-dependent mechanisms. How such enhanced activity reduces approach to the male incentive is not entirely clear, but some speculations can be made. We have earlier shown that approach to the male is mainly determined by social motivation. For example, manipulations altering sexual motivation, like castration or extensive sexual activity immediately preceding the test, do not modify approach behaviors to the male (Ågmo, 2003; Ågmo et al., 2004). Others have also shown that male rats approach other males (Eckman et al., 1969; Latané, 1969; Latané et al., 1972, 1973; Latané and Glass, 1968), and that this social approach is independent of immediately preceding sexual activity (Sloan and Latané, 1974). In view of this it is likely that the reduced approach to the male seen after Impaza, 3 ml and sildenafil is a result of lowered social motivation.

This proposal is substantiated by the fact that the time spent in the male incentive zone after Impaza, 3 ml and sildenafil is not larger than the time the experimental subjects would have spent there if their position in the arena were random. A calculation of the time the males should be expected to spend in the incentive zones if their position indeed were random gives 83 s ((incentive zone surface/total arena surface) × test duration). This is superior to the value obtained in the group treated with Impaza, 3 ml, in which the experimental males spent 44 ± 13 s (mean ± SE) in the male incentive zone. In fact, this value is significantly different from the expected value of 83 s ($t_8=2.89$, $P<0.05$). It appears, then, that the males treated with Impaza, 3 ml actively avoided the incentive male. In the group given sildenafil there was no difference between actual and theoretical random time in the male incentive zone (88 ± 27 and 83 s, respectively; $t_8=0.86$, NS), suggesting that these males neither approached nor avoided the male incentive. For comparison, it

may be mentioned that the control males spent more time in the male incentive area than random position would predict (t_{44} = 46, t = 3.42, P<0.05). This coincides with earlier data showing that male subjects spend more time in the vicinity of another male than in the vicinity of an empty incentive cage (Ågmo et al., 2004). Furthermore, this observation confirms that male rats are socially attracted to other males.

The arguments exposed in the preceding paragraph suggest that sildenafil abolished social motivation while Impaza, 3 ml, not only eliminated the incentive male's social incentive value, but also turned him into a negative incentive, producing withdrawal. These effects could, in principle, be explained by an anxiogenic action of Impaza and in minor degree of sildenafil. A study performed in male Fisher 344 rats shows that anxiogenic compounds reduce the time spent close to an inaccessible social incentive (a male rat) whereas anxiolytic compounds enhance it (Nicolas and Prinssen, 2006). Indeed, a reduced time close to a social incentive was exactly what was observed in the present experiment. However, data are contradictory with regard to the effects of nitric oxide and cGMP on anxiety (see Guimaraes et al., 2005 for a review). Some effects have been reported in the elevated plus-maze, but the few studies employing the social interaction test for anxiety have reported mixed effects of altered availability of nitric oxide. For example, Volke et al. (1997) reported enhanced social interaction following treatment with the nitric oxide synthase inhibitor 7-nitroindazole. However, a study employing another nitric oxide synthase inhibitor, NAME, did not find any effect at all on social interaction (Vale et al., 1998). The effects of manipulations of cGMP concentrations with sildenafil on anxiety-like behaviors have not been much studied, but the majority of data suggests an anxiogenic action (Kurt et al., 2004; Volke et al., 2003). Unfortunately, the data stem exclusively from mice, and there is no study with regard to effects on social interaction. Nevertheless, it does not seem unreasonable to suggest that the reduced social motivation observed after treatment with sildenafil or Impaza, 3 ml may be related to anxiogenic effects of enhanced activity in the nitric oxide–cGMP pathway. Additional experiments are needed in order to determine how nitric oxide-dependent mechanisms modify social motivation.

An interesting consequence of the reduced social motivation displayed by the animals treated with sildenafil and particularly with Impaza, 3 ml combined with the fact that the time spent in the female incentive area was not reduced, is that the female's sexual incentive value must have been enhanced by these treatments. Considering that the female is both a social and sexual incentive, reduced social motivation must have been compensated for by an increase in sexual motivation. Otherwise, the time spent in the female incentive area should have been reduced. Thus, it can be speculated that sildenafil and especially Impaza, 3 ml, enhanced the female's sexual incentive value. However, only further studies in other procedures can substantiate this proposal. Nevertheless, the data obtained in the experiment reported here are sufficiently suggestive to justify such studies.

None of the behavioral effects observed after treatments with sildenafil and Impaza can be attributed to altered general activity. All activity indices failed to detect any difference between treatments. It is noteworthy, though, that a group of younger Fisher 344 males had a much higher activity than the old males employed in the experiment. This observation suggests that reduced sexual activity is only one of many behavioral changes occurring with advancing age.

At present, the pharmacokinetics and pharmacodynamics of Impaza are entirely unknown. However, the data mentioned in the Introduction show that it does stimulate eNOS activity and that it constitutes an efficient proerectile treatment. Since eNOS is located in blood vessels, there is no need for the compound to cross the blood-brain barrier in order to be active. Nevertheless, it needs to be absorbed from the intestines and enter the circulation. The exact mechanisms participating in these processes are not known. Incomplete knowledge of the mechanisms of action is not unusual among clinically active compounds, though.

In sum, the data obtained in the present experiment show that old male rats fail to approach a sexually receptive female more than another male. Furthermore, sexually naive old males show very little copulatory behavior when given access to a sexually receptive female. Stimulation of eNOS may enhance sexual incentive motivation in these old rats without activating copulatory behavior. The PDE5 inhibitor sildenafil had a borderline effect on incentive motivation and none on copulatory behavior. The effects on social motivation observed in the present experiment are difficult to explain, but they suggest that nitric oxide–cGMP dependent mechanisms are among the many central nervous mechanisms determining social incentive value. Present results show that proerectile compounds acting through the nitric oxide–cGMP system may have important effects on motivational processes.

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